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UNIVERSITY OF ALBERTA

Racemic Pharmaceuticals: Analysis and Pharmacokinetics of Flecainide and Methocarbamol Enantiomers

by

Silvia Alessi-Severini

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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To my husband Alberto and my children Giorgia and Giulia.

ABSTRACT

It has become evident in recent years that the investigation of racemic drugs must include the evaluation of the pharmacokinetics of the enantiomers; as a consequence, the development of stereospecific analytical methods for the determination of plasma and urine levels in animal models and humans is of utmost significance.

An HPLC assay for the determination of the enantiomers of the antiarrhythmic flecainide was developed. After extraction, racemic flecainide was derivatized with an optically pure reagent (1-[(4-nitrophenyl) sulphonyl]-L-prolyl chloride). Separation of the diastereomeric derivatives was obtained on a reverse phase column using a water:acetonitrile mobile phase (55:45). UV detection was carried out at a wavelength of 280 nm. Interday CV ranged from 3.9 % to 9.6%). The sensitivity was 50 ng/mL for each enantiomer. The pharmacokinetic patterns of the two enantiomers were found to be similar. An exellent correlation was found between this method and the non-stereospecific fluorescence polarization immunoassay (r=0.96). Stereoselective therapeutic monitoring data were generated for 23 patients. Plasma R/S ratios ranged from 0.75 to 1.33.

A second stereospecific HPLC method was developed for racemic methocarbamol, a widely used muscle relaxant This assay consisted of extraction of racemic methocarbamol and derivatization for 12 h at 85 °C with (S)-(+)-1-(1-naphthyl) ethyl isocyanate. Separation of the diastereomeric derivatives was obtained on a normal phase column using a 95:5 hexane:isopropanol mobile phase. UV detection

was conducted at 280 nm. Interday CV ranged from 3.9% to 9.8%. The sensitivity was determined to be 0.5 μg/mL for each enantiomer. This assay was applied to a study of the pharmacokinetics of methocarbamol enantiomers in the rat. S-methocarbamol was generally predominant in plasma with significantly lower plasma clearance. Both enantiomers showed linear pharmacokinetics and a reduced oral bioavailability. First-pass metabolism in the intestine was suggested. Protein binding was low (40% for each enantiomer). Urinary excretion of the unchanged was less than 10 % for both enantiomers).

Plasma profiles of methocarbamol enantiomers were also determined in humans after administration of a single oral dose of 2 g of racemic methocarbamol. Similar to the situation with the animal model, S-methocarbamol was predominant in plasma, with oral clearance significantly lower than the corresponding value for R-methocarbamol. Two known poor metabolizers of debrisoquine showed elevated plasma levels for both enantiomers as compared to extensive metabolizers.

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GLOSSARY OF ABBREVIATIONS

4-OHDQ	4-hydroxydebrisoquine
α	separation coefficient
ANOVA	analysis of variance
AUC	area under plasma concentration-time curve
AUC_{ip}	AUC after ip administration
AUC_{iv}	AUC after iv administration
AUC_o	AUC after oral administration
AUMC	area under the first moment curve
CAST	cardiac arrhythmias suppression trial
CD	
CL_o	oral clearance
CL_s	systemic clearance
C_{max}	maximum plasma concentration
CV	coefficient of variation
E	error percent
EM	extensive metabolizer
eV	electron volt
F	oral bioavailability
FPIA	fluorescence polarization immunoassay
g	grams
GC	gas chromatography
GI	gastrointestinal tract
h	hours
HPLC	high-performance liquid chromatography

ip	intraperitoneal
IS	internal standard
iv	intravenous
kg	kilogram
L	litre
LC	liquid chromatography
µg	micrograms
mg	milligrams
mg/L	milligrams per litre
min	minutes
mL	milliliter
MODE	3-methoxy-O-desmethylencainide
MS	mass spectrometry
n	number of observations
NEIC	(S)-(+)-1-(1-naphthyl)ethyl isocyanate
ng	nanograms
NMR	nuclear magnetic resonance
NPSPC	1-[(4-nitrophenyl)sulphonyl]-L-prolyl chloride
ODE	O-desmethylencainide
p	level of significance
PM	poor metabolizer
po	oral
r	correlation coefficient
RBEIT	R-1-(β-naphthyl)ethyl isothiocyanate
R_s	resolution factor
SD	standard deviation

sec	seconds
$t_{1/2}$	half-life
TAGIT	tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate
TD_X	Abbott's FPIA detection system
TEA	triethylamine
t_{max}	time to peak maximum plasma concentration
TPC	N-trifluoroacetyl-L-prolyl chloride
UV	ultraviolet
V_d	volume of distribution
$V_{d_{ss}}$	volume of distribution at the steady-state
X_u^{∞}	cumulative urinary excretion to time infinity

Preamble

The importance of pharmacokinetic and pharmacodynamic investigations of racemic drugs has become evident in recent years. A pioneer paper by Ariens in 1984 [1] addressed the issue of stereoselectivity in drug action and disposition and highlighted the possibility of therapeutic and pharmacokinetic "nonsense" in cases where stereoselective information is ignored. The study of the properties of the enantiomers in drugs administered as racemates is now considered essential for a complete understanding of the biological fate as well as the therapeutic effectiveness of such drugs. There are numerous drugs which are administered as racemates and show significant differences in enantiomer pharmacological activity, pharmacokinetics, metabolism and toxicity.

It is, in fact, more common to find that one enantiomer of a racemic mixture is more potent than its antipode than it is to have equipotent and equiactive isomers [2]. The antiinflammatory 2-arylpropionic acids and etodolac owe their cyclooxygenase inhibitory activity to the S-enantiomer, whereas the R-isomer is practically inactive [3]. Similarly, S-propranolol is about 100 times more active as a beta-blocker than the R-enantiomer [4], warfarin anticoagulant activity resides mostly in the S-antipode [5], and the S-enantiomer of the calcium channel blocker verapamil is ten times more potent than its optical isomer[2]. The antihypertensive labetalol possesses two chiral centers, and, as a consequence, exists as four stereoisomers. The drug is administered as an approximately equimolar mixture of the two racemates and shows a complex pharmacological profile: the

(R,R)- isomer is responsible for most of the β−blocking activity and has shown some β_2-agonist activity, while the (S,R)- isomer is an α−adrenoceptor antagonist. The S,S- and the R,S- forms are essentially inactive [6].

Stereoselectivity in disposition and metabolism complicates the interpretation of pharmacokinetic data in relation to therapeutic effects of racemic pharmaceuticals. Absorption and distribution are generally non-stereoselective processes regulated by passive diffusion. However, in a few instances active transport is involved and, in fact, only the L-forms of methotrexate [7] and dopa [8] are actively transported through the gastrointestinal wall. Furthermore the R isomers of the 2-arylpropionic acids are selectively incorporated into adipose tissue as a result of the formation of R-coenzyme A thioesters (which replace the fatty acid moieties in natural triglycerides) [2]. There are few reports on stereoselective tissue binding [2], whereas plasma protein binding of enantiomers has been extensively studied in recent years. An interesting example is disopyramide, the two enantiomers of which show similar pharmacokinetics when measurements of individual enantiomers are considered. However, clearance of the unbound S-enantiomer is significantly higher than that of R-disopyramide; this discrepancy is explained by the more extensive plasma protein binding of S-disopyramide [2].

Renal elimination can show stereoselectivity in cases of stereoselective protein binding because glomerular filtration depends upon the free fraction. Of course, active tubular secretion can be stereoselective as is the case for quinidine [9].

On the other hand, stereoselectivity is routinely encountered in Phase I and Phase II metabolic reactions [2,10], and there are numerous examples of drugs that show substrate stereoselectivity (preferential enzymatic metabolism of one enantiomer) [2]. An interesting example of stereoselective metabolism is represented by the chiral inversion observed with 2-arylpropionic acids, from the inactive R- to the active S-enantiomer [11]. Another important example is stereoselective first-pass metabolism which can occur in both GI tract and liver. Most β-blockers undergo significant first-pass metabolism after oral administration [2]. A typical example is propranolol, whose R-isomer shows a higher intrinsic clearance than its antipode. Since both enantiomers present a very high extraction ratio, the difference in intrinsic clearance produces a substantial difference in enantiomer bioavailability, but their plasma-concentration profiles are superimposable after intravenous administration [12]. A similar case is presented by verapamil, where the less active R-isomer shows a five-fold greater concentration in plasma than its antipode after oral administration of the racemate [13]. Interesting, but less common, are cases of product- and substrate-product stereoselectivity where a prochiral drug is preferentially metabolized to one of the two possible enantiomers (e.g. phenytoin to (S)-5-(4-hydroxyphenyl)-5-phenylhydantoin [2]) and a drug which possesses both a chiral and a prochiral centre undergoes stereoselective metabolism and generates another chiral centre (e.g. perhexiline [2]).

Oxidative polymorphism is a special aspect of stereoselective metabolism and is determined by a deficiency of some P450

enzymes in certain percentages of the population. Of particular interest are the P450IID6 isozyme which determines the sparteine-debrisoquine phenotype (5-10% incidence of deficiency in Caucasians) [14] and the P450IIC9 isozyme which may control the mephenytoin phenotype (2-4% in Caucasians, 22% in Orientals) [15]. The individuals who express the enzyme are classified as extensive metabolizers (EM), whereas those who are deficient are called poor metabolizers (PM). The significance of genetic polymorphism on the pharmacokinetics of, and pharmacological response to, those drugs that cosegregate with the test probes is understandable. Stereoselectivity is also a prominent aspect in the determination of the phenotype. Debrisoquine, for example, is an achiral drug whose metabolic 4-hydroxylation in EMs leads exclusively to the S(+)-4-hydroxydebrisoquine (4-OHDQ); in PMs, 5-36% of the total 4-OHDQ is excreted as the R-enantiomer [16]. Encainide, a racemic class Ic antiarrhythmic, is stereoselectively metabolized to ODE (O-desmethylencainide) and MODE (3-methoxy-O-desmethylencainide) by EMs, whereas stereoselectivity is not observed in PMs. PMs also do not produce any MODE. Cytochrome P450IID6 activity is enantioselective for (-)-encainide [17].

Mephenytoin is a racemic anticonvulsant whose enantiomers undergo stereoselective metabolism. The S-isomer is selectively hydroxylated and rapidly eliminated, whereas the R-isomer undergoes a slow non-stereoselective demethylation to the active metabolite nirvanol. Those individuals who are deficient in the hydroxylation pathway eliminate both enantiomers as nirvanol. The phenotype is determined by the S/R urinary ratio; a value close to 1

will reveal a PM, while a ratio significantly lower than 1 will determine an EM [15].

There is some evidence that stereoselective production of toxic metabolites is possible. For example, there is speculation that the optic-neuritis caused by penicillamine is mediated by the L-isomer, while only D-dopa seems to induce granulocytopenia [18]. Furthermore, the teratogenic activity of thalidomide seems to arise from the S-enantiomer which is tranformed *in vivo* to l-N-phthaloylglutamine and l-N-phthaloylglutamic acid, products that have shown embryotoxic and teratogenic effects in some animal models (SWS mice and Natal rats) [19]. More recently some doubts have been raised regarding the extrapolation of these results to primates and humans [20].

In light of the pharmacokinetic and therapeutic significance of a stereospecific approach to drug investigation, the development of analytical methods which allow chromatographic discrimination between the enantiomers becomes crucial as a first step toward a complete understanding of the biological fate of new racemic drugs. The re-investigation of old racemic drugs is also significant to update and expand the pharmacokinetic knowledge of these drugs.

There are two basic approaches to chromatographic enantioseparation: an indirect method based on derivatization of the racemate with an enantiopure reagent and separation of the obtained diastereoisomers on a "conventional" achiral GC or HPLC column, and a direct method with the utilization of chiral columns under GC or HPLC conditions. The addition of chiral mobile phase additives to HPLC solvents may also be useful [21-24].

There are numerous reagents which are commercially available at a satisfactory level of chemical and optical purity for the derivatization of primary and secondary amines, alcohols, phenols and caboxylic acids. The most popular are the isocyanates and isothiocyanates which can increase UV and fluorescence properties of a drug. Chloroformates and prolyl chlorides are also widely used [21-24]. Fluorinated prolyl chlorides represent a first choice to increase volatility and sensitivity to electron-capture detection in GC.

There are several chiral stationary phases available for GC. They can be divided into three groups: the amino-acid phases, such as L-valine-tert-butylamide, the cyclodextrin phases and the metal complex phases. The resolution of the enantiomers is based on differential stereoselective interactions between the enantiomer and the chiral environment offered by the stationary phase.

High performance liquid chromatography appears to be more versatile and shows a larger choice of chiral stationary phases; these include crown-ether phases, the $\pi-\pi$ interaction phases where generally a dinitrobenzoyl-derivatized phenylglycine or leucine, linked to a silica support, operates as a $\pi-$electron acceptor and stereoselectively attracts $\pi-$electron donating aromatic systems; other packings include α_1- acid glycoprotein and bovine serum albumin phases, as well as cyclodextrins, polysaccharides and helical methacrylate polymers [21-24].

The use of chiral mobile phase additives has also been reported: some of these include (+)-0-camphorsulfonic acid, quinidine and N-benzoxycarbonyl-glycyl-L-proline as well as cyclodextrins [21-24] .

In this myriad of choices, it is a challenge for the analyst to find the right solution and the best conditions for every individual separation.

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1. FLECAINIDE

1.1. Introduction*

1.1.1. History

(R,S)-Flecainide, which is administered as an acetate salt, is a class 1c antiarrhythmic agent which was developed in the Riker Laboratories as part of a broad-based project investigating the effect of fluorine substitution on local anaesthetic or antiarrhythmic activity [1]. It was introduced on the market in 1985 as a promising agent in the treatment and control of ventricular and supraventricular arrhythmias. Despite alarming cases of fatal toxicity [2,3], the drug became widely prescribed in many different conditions of ventricular and atrial arrhythmias. In 1989 the results of a multicentre investigation on several antiarrhythmics (CAST, Cardiac Arrhythmias Suppression Trial) showed unequivocally that patients recovering from myocardial infarction and exhibiting asymptomatic ventricular arrhythmias, when treated with flecainide, suffered a mortality rate twice as high as those treated with placebo [4]. At that point flecainide became a restricted drug to be used only in cases of life-threatening arrhythmias, such as sustained ventricular tachycardia.

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1.1.2. Physicochemical properties

Molecular weight

flecainide free base $C_{17}H_{20}F_6N_2O_3$ = 414.36.

flecainide acetate $C_{19}H_{24}F_6N_2O_5$ = 474.40

Appearance, color and odor

Flecainide free base: white granular solid when crystallized from isopropyl alcohol/isopropyl ether. Odorless. Acetate: white crystalline solid.

Optical rotation and absolute configuration

Optical rotations (sodium D line, 1 dm cells, methanol as solvent) were obtained with a Perkin Elmer Model 241 polarimeter. Optical purity of flecainide free base enantiomers was >90% as determined by 100 MHz NMR using the chiral shift reagent tris[3-heptafluorobutyryl-d-camphorato]-europium III [5].

(+)-flecainide free base $[\alpha]^{26}_D$ +3.4°

(-)-flecainide free base $[\alpha]^{26}_D$ -3.3 °

(+)-flecainide acetate $[\alpha]^{26}_D$ -4.6°

(-)-flecainide acetate $[\alpha]^{26}_D$ -4.5°

The absolute configurations of flecainide enantiomers have been determined on the basis of CD spectra of the N-chloro derivatives. Thus, (+)-flecainide has the S-configuration while the antipode is R-(-)-flecainide [6].

Melting point

(R,S)-flecainide free base 105°-107° C

(+)-flecainide free base 104-105° C [5]

(-)-flecainide free base 102-104° C[5]

(R,S)-flecainide acetate 145°-147° C [7]

(+)-flecainide acetate 153-155° C [5]

(-)-flecainide acetate 152.5- 154°C [5].

Ionization constant

The pK_a of flecainide acetate has been determined as 9.3 [8].

Distribution coefficient

The octanol/water partition coefficient was determined to be 11.4 at pH 7.4 [9] and logP calculated as 4.50 [10].

Solubility

The solubility of flecainide acetate in water at 37°C is 48.4 mg/mL in water and 300 mg/mL in alcohol [8].

Stability

A solution of flecainide acetate in water has been reported to be very stable at room temperature [11]. The stability in biological fluids seems to be significantly decreased (30-40%) over a period of 3 months even under storage at -20°C [12].

1.1.3. Pharmacodynamics, pharmacokinetics and metabolism

The pharmacodynamics and pharmacokinetics of (R,S)-flecainide acetate have been studied extensively in animal models and in humans. This drug exhibits potent antiarrhythmic effects by blocking the cardiac sodium channels and by stabilizing the myocyte membrane without affecting repolarization [13-17]. It is used in the suppression and control of ventricular and supraventricular arrhythmias [13-19] and has shown some effectiveness in the treatment of atrial arrhythmias [20-22]. Because of life-threatening toxicity the drug is not recommended in non-symptomatic post-infarction patients [23].

Flecainide acetate is rapidly and almost completely absorbed from the GI tract following oral administration. Absolute bioavailability of the commercially available tablets averages 85-90%. First pass metabolism is negligible. Plasma concentrations must be kept in the 200-1000 ng/mL range in order to maximize the therapeutic effect and minimize the risk of serious side effects [13-17]. Plasma levels and dose generally show a linear correlation [24], however considerable interindividual and intraindividual variations have been observed and, more recently, evidence of non-linear kinetics has been reported [25]. After iv administration to humans, flecainide is rapidly and apparently widely distributed (V_d ranges from 5 to 13.4 L/kg, average: 5.5-8.7 L/kg). After oral dosing V_d has been evaluated to be 10 L/kg [26]. After iv administration to rats, flecainide is distributed extensively to many tissues, including the

heart, but only minimally into the CNS. Dose-dependent tissue uptake has been shown in rabbits after chronic administration [27,28]. The *in vitro* protein binding (mainly to α_1–acid glycoprotein) of flecainide is approximately 40-50% and is independent of the plasma concentration of the drug [13-17]. The pharmacokinetic profile after iv administration follows a 2-compartment open model with $t_{1/2\alpha}$ =3-6 minutes and $t_{1/2\beta}$ = 11-14 hours (range 7-19 h) [26]. Elimination half-life is prolonged in arrhythmias (19 h), in renal and hepatic impairment (26-49 h), and in congestive heart failure (up to 50 h) [26,29-34]. Urinary pH affects elimination half-life, prolonging it when very alkaline (pH 7.2-8.3) and reducing it when very acidic (pH 4.4-5.8) [35-37].

Flecainide is metabolized by the liver. The two major metabolites are m-O-dealkylated flecainide and the m-O-dealkylated lactam derivative. These are formed by preferential O-dealkylation at the meta position of the benzamide ring and by subsequent oxidation of the piperidine ring of m-O-dealkylated flecainide, respectively. Both metabolites are extensively conjugated with glucuronic and sulfuric acid at the m-O-dealkylated position. About 80-90% of a dose is recovered in the urine (30% as unchanged drug, the rest as metabolites and their conjugates). Only 5% is excreted in the feces and 3% of the total excretion is attributed to at least 3 unidentified metabolites [26]. Hemodialysis removes about 1% of a dose as unchanged drug. Total apparent plasma clearance of flecainide has been reported for healthy subjects, following oral administration, to be in the range 4-20 mL/min/kg. Renal clearance of the drug is 25-40% of total plasma clearance. Total apparent plasma clearance is

decreased in arrhythmias, congestive heart failure and renal impairment [13-17, 29-32, 34].

There is comparatively little information regarding stereoselectivity in pharmacodynamics, pharmacokinetics, and metabolism. *Ex vivo* studies have revealed equipotency and equiactivity for the two enantiomers in mouse and dog models of arrhythmia and they have shown similar electrophysiologic effects in humans [5, 38]. The two enantiomers have also shown similar affinities to a receptor site associated with cardiac sodium channels in isolated rat cardiac myocytes [39].

Pharmacokinetic investigation of flecainide enantiomers, after oral administration of the racemate, has been conducted only recently following the development of stereospecific analytical methods [40-42]. Our findings are discussed in detail in chapters 1.2 and 1.3.

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ion chemical ionization and stable isotope labelled internal standards. *Biomed Environ Mass Spec* **19** 256-266 (1990).

1.2. High-performance liquid chromatographic determination of the enantiomers of flecainide in human plasma and urine*

1.2.1. Introduction

(R,S)-Flecainide acetate [(R,S) - N-(2-piperidylmethyl)-2,5-bis-(2,2,2-trifluoroethoxy) benzamide acetate, Figure 1.2.1.] was the first Class 1c antiarrhythmic released in North America. Drugs included in this subgroup of membrane-stabilizing agents exhibit potent blocking effects on cardiac sodium channels but leave repolarization relatively unchanged. (R,S)-Flecainide is effective in various age groups [1] for the control of superventricular and ventricular arrhythmias, as well as refractory ventricular tachycardia [2]. Its usefulness in atrial arrhythmias is not well established and its role in patients with congestive heart failure continues to be assessed [3]. The clinical pharmacology and therapeutic uses have been reviewed and a risk:benefit analysis in which flecainide was compared to other antiarrhythmics has been presented [5].

A variety of issues related to enantioselective action and disposition of racemic drugs has drawn considerable interest in recent years. There is merit, for example, to questions concerning the relevance of pharmacological, pharmacokinetic, and therapeutic drug

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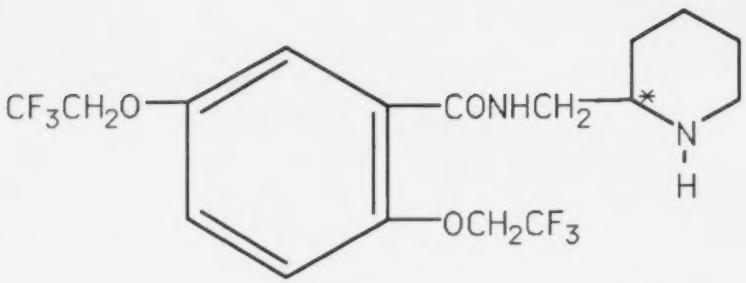

Figure 1.2.1. Structure of (R,S)-flecainide. The asterisk indicates the chiral centre

monitoring data obtained from assays which do not discriminate between enantiomers [6-10]. Although flecainide is administered as a racemate, there is limited information concerning the pharmacological properties of the enantiomers. *Ex vivo* studies have revealed that the isomers are essentially equiactive in mouse and dog models of arrhythmia [11], while a rat cardiac myocyte binding assay was used to show that the enantiomers also had similar affinities to a receptor site associated with cardiac sodium channels [12].

Although numerous analytical methods for (R,S)- flecainide have been published [13-25], there are few reports describing enantioseparation [26,27]. The purpose of this study was to develop a stereospecific assay which could be applied to a preliminary investigation of the pharmacokinetics of flecainide enantiomers in humans. Three other reports of stereoselective investigation on flecainide were published in parallell with ours [28-30]. All the findings will be addressed in the conclusion of this chapter (section 1.4).

1.2.2. Experimental Section

1.2.2.1. Chemicals and reagents

The internal standard [IS, (R,S)-N-(2-piperidylmethyl)-2,3-bis-(2,2,2-trifluoroethoxy)benzamide hydrochloride Figure 1.2.2.], racemic flecainide acetate, and both flecainide acetate enantiomers were gifts from Selvi 3M (Milan, Italy) and Riker Laboratories (St. Paul, MN). The optically pure derivatizing reagent, 1-[(4-

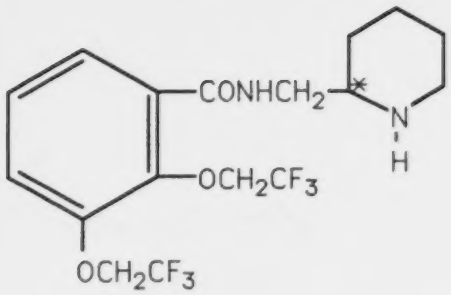

Figure 1.2.2. N-(2-piperidylmethyl)-2,3-bis-(2,2,2-trifluoroethoxy)benzamide (Internal standard).

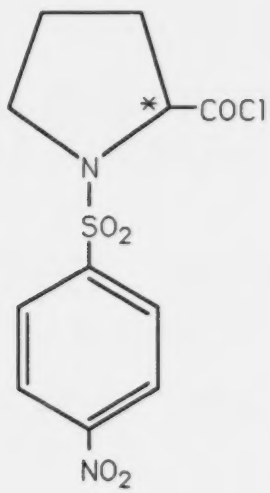

Figure 1.2.3. 1-[(4-nitrophenyl)sulphonyl]-L-prolyl chloride (Derivatizing agent). The asterisk indicates the chiral centre.

nitrophenyl)sulphonyl]-L-prolyl chloride (NPSPC) (Figure 1.2.3.), was synthetized from L-proline (99+% purity) according to a published procedure [31] and gave physical data consistent with reported values. Diethyl ether and ethyl acetate were obtained from BDH Chemicals (Edmonton, Canada) and glass distilled prior to use. Reagent grade triethylamine (TEA) was purchased from Fisher Scientific (Edmonton, Canada) while HPLC grade acetonitrile and water were obtained from Mallinckrodt (Canlab, Edmonton, Canada). A stock solution of (R,S)-flecainide acetate was prepared by dissolving 10 mg in 100 mL of water. Working solutions of blank plasma and urine were obtained by appropriate dilution and spiked to give final flecainide enantiomer concentrations of 25, 50, 125, 250, and 500 ng/mL. A 4 μg/mL aqueous working solution of the IS was prepared by diluting the 0.1 mg/mL stock solution. The 0.049M solution of NPSPC in ethyl acetate was freshly made prior to use, and a working solution of TEA was prepared by diluting 0.004 mL in 25 mL of distilled ethyl acetate.

1.2.2.2. Instrumentation

The HPLC system (Waters Scientific, Mississauga, Canada) consisted of a 590 pump, a 481 variable wavelength UV detector set at 280 nm, a 710B Wisp autosampler, and a 3390A integrator-recorder (Hewlett Packard, Mississauga, Canada). Direct - inlet mass spectra were recorded on a 5980A mass spectrometer, 5934A data system (Hewlett Packard) using an electron energy of 70 eV and source temperature of 180°C. Samples were mixed with an IKA vibrax VXR mechanical shaker (Terochem, Edmonton, Canada) and

centrifuged in a Clay-Adams centrifuge (Parsippany, NJ). Solid- phase extraction was accomplished on a Supelco SPE Vacuum Manifold using disposable 1 mL C_8 extraction columns (Supelclean, LC-8, Supelco, Oakville, Canada). During derivatization, samples were heated in a Reacti-Therm block (Pierce Chemicals, Rockford, IL).

1.2.2.3. Chromatographic conditions

The Waters μ-Bondapak C_{18} reverse - phase column (3.9 mm x 30 cm) was preceded by a 5-cm guard column packed with octadecyl Pellicular ODS (Whatman, Clifton, NJ). The mobile phase consisted of acetonitrile: water: TEA (45:55:0.2) and was pumped at a flow rate of 1 mL/min. The fused silica Chirasil-Val gas chromatographic column was obtained from Terochem (Edmonton, Canada) while the DB-1, DB-5 and DB-17 columns were purchased from J & W Scientific (Rancho Cordova, CA).

1.2.2.4. Sample analysis

Due to concerns about stability (pg. 13), all samples were analyzed immediately upon receipt. After addition of 0.1 mL of IS solution, 1 mL of water and 1 mL of 1M NaOH, the constituents of 1 mL of plasma (or diluted urine 1:100) were shaken with 8 mL of distilled diethyl ether for 15 min. The samples were then centrifuged at 1000 x g for 5 min and, after transfer to clean tubes, the organic layer was evaporated to dryness under a stream of nitrogen. Solid-phase extraction was also employed for comparison purposes. Samples were processed according to a reported method [19] except that they were adjusted to pH 11 (with 0.01M NaOH) rather than pH

9 prior to extraction. The constituents were eluted with 1 mL of methanol and the samples evaporated to dryness. To the residue remaining were added 1 mL of NPSPC solution followed by 0.1 mL of TEA solution. The tubes were briefly vortexed (5 sec) and heated at 80°C for 2h. After cooling, the samples were washed with 3 mL of 0.6 M HCl and centrifuged for 5 min. The organic phase was transferred to clean vials, evaporated to dryness under a stream of nitrogen, and the residues reconstituted in 0.2 mL of mobile phase. Aliquots of 0.04-0.18 mL were injected into the HPLC apparatus.

1.2.2.5. Accuracy and precision

The accuracy and precision of the method were evaluated by analyzing four replicate spiked plasma samples at each of five concentrations against a calibration curve. The difference between mean estimated and mean added concentrations was taken as the accuracy of the method, while precision was estimated by calculating the intra- and inter-day coefficients of variation.

1.2.2.6. Patient sample collection

The project was conducted in accordance with the principles of the Declaration of Helsinki. The 39-year-old female patient was being treated for atrial tachycardia and had been receiving 100 mg of (R,S)-flecainide three times daily for 6 weeks. Blood samples were obtained just before the first morning 100 mg dose, and then after 30 min and 1, 1.5, 2, 3, 4, and 5 h. Specimens were centrifuged and plasma was separated prior to analysis. Total urine output was collected for only 6 h after drug administration.

1.2.3. Results and Discussion

Initial efforts were directed towards the development of a gas chromatographic assay since the fluorine atoms present in (R,S)-flecainide favor volatility and sensitivity to electron-capture detection. To minimize column adsorption and peak tailing, the secondary amine was derivatized with pentafluorobenzoyl chloride, according to a published procedure [13], and the products chromatographed on a Chirasil-Val column. The derivatized enantiomers failed to resolve regardless of the column conditions employed. Diastereomer formation with other acid chloride reagents including (-)-camphanic acid chloride, (-)-α-chlorophenylacetyl chloride and 1-[(4-nitrophenyl) sulfonyl]-L-prolyl chloride, as well as R-(+)-1-phenylethyl isocyanate and S-(-)-1-(1-naphthyl) ethyl isocyanate, followed by chromatography on achiral capillary columns (DB-1, DB-5, and DB-17), was similarly unsuccessful. In each case it appeared that the derivatization reaction had gone to completion since unreacted (R,S)-flecainide was not observed in the chromatographic traces. Gas chromatography was subsequently abandoned due to lack of resolution or insufficient volatility and/or stability of the derivatives.

(R,S)-Flecainide has been resolved by normal-phase HPLC following derivatization with R-(+)-1-phenylethyl isocyanate (separation coefficient, α, of 1.19, resolution factor, R_S, of 2.37)[26]. This study, however, did not present an analytical method; the diastereomers were prepared on a milligram scale in order to determine the optical purity of the respective enantiomers. Although

the procedure was subsequently reproduced in our laboratories, the poor reaction yield and low sensitivity precluded the use of this method for the assay of biological samples. Some degree of resolution has also been achieved using various homochiral isothiocyanates including tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT), S-1-(α-naphthyl) ethyl isothiocyanate, R-1-(β-naphthyl) ethyl isothiocyanate (RBEIT), and R-1-phenylethyl isothiocyanate [27]. The α values varied from 1.00 for RBEIT to 1.16 for TAGIT. Although the largest α value was observed with TAGIT, the resolution factor was not provided and it is unclear whether this degree of separation is sufficient to be useful for pharmacokinetic studies [32].

Secondary amines react readily with L-proline-based reagents including N-heptafluorobutyryl-L-prolyl chloride [33] and N-trifluoroacetyl-L-prolyl chloride (TPC) [34]. They were not selected for the present study since both are more suitable for GC analysis and TPC must be freshly prepared as it is prone to racemization [35]. Attention was therefore directed to 1-[(4-nitrophenyl) sulfonyl]-L-prolyl chloride since the GC studies conducted earlier confirmed that it reacted with (R,S)-flecainide. Furthermore, it was a readily synthesized, recrystallizable solid which possessed favorable UV absorption properties [31]. Previous observations that the reagent does not undergo significant racemization [31] have been confirmed; reaction with individual enantiomers gave a single chromatographic peak in each instance. Although stable as a solid, an ethyl acetate solution of the reagent was observed to decompose over a 2-day period. The solution was therefore freshly prepared prior to use.

Flecainide was extracted from plasma by either solid-phase or solvent extraction. The reported recovery by solid phase extraction ranges from 73% [15] to 103% [20]. The efficiency of extraction obtained was consistently > 90%. The solvent method, however, was chosen since a simple single-step extraction process with diethyl ether gave recoveries comparable to the solid-phase process. The interference and solubility problems reported by Chang *et al.* [15] with respect to the use of diethyl ether as extraction solvent, were not experienced under our experimental conditions and this solvent was found to be superior to dichloromethane, ethyl acetate, or hexane.

The reaction of NPSPC with flecainide enantiomers was conducted in ethyl acetate although dichloromethane or acetonitrile gave equally acceptable results. Direct reaction with NPSPC in aqueous media, as reported for the derivatization of amphetamine [36], was also attempted in order to improve sample processing. This approach was unsuitable due to co-extraction (after the *in situ* derivatization) of several interfering substances which subsequently appeared in the chromatograms. The reaction in ethyl acetate was complete after heating at 80°C for 2 h. There was no evidence of unreacted flecainide, which had a retention time of 14 min under our chromatographic conditions. A final acid wash was added to the procedure in order to remove an endogenous substance which gave an interfering peak in the chromatograms. Since kinetic resolution was observed when the reaction was conducted at room temperature, the derivatization conditions described above were necessary in order to avoid stereoselective formation of

diastereomers. A preferential reaction with S- flecainide was evident by the predominant formation of the corresponding diastereomer. Interestingly, this asymmetric bias was also evident with the IS, a positional isomer of flecainide which was purposely selected in order to minimize the effect of experimental error during sample preparation. This observation proved useful since a 50:50 peak area ratio for the derivatized IS enantiomers confirmed that the derivatizing reaction had gone to completion without asymmetric induction. The mass spectrum of derivatized (R,S)-flecainide lacked a molecular ion (m/z 696) but contained six abundant fragment ions; m/z (% relative abundance): 366 (44), 301 (56), 255 (100), 186 (21), 122 (22), and 84 (25). Three of these ions were of particular diagnostic value in confirming the structure of the diastereomeric flecainide derivatives. Appropriate structures for these fragment ions are suggested in Figure 1.2.4. The separation of flecainide diastereomers on the reverse-phase column was satisfactory (α = 1.07, R_s = 1.25). Addition of TEA to the mobile phase resulted in sharp, well-resolved chromatographic peaks despite the relatively long retention times of 29.8 min for R-flecainide and 31.9 min for S-flecainide diastereomers (Figure 1.2.5). The order of elution was confirmed by comparison with similarly derivatized optically pure enantiomers. The IS diastereomers were also well resolved (α = 1.09, R = 1.67) with retention times for R and S enantiomers of 25.5 min and 27.9 min, respectively (Figure 1.2.5.). Blank samples did not show any interfering peaks in the analytical region of the chromatograms. For the sake of consistency, the first eluting peak of the IS was used for quantification and a very good linearity was

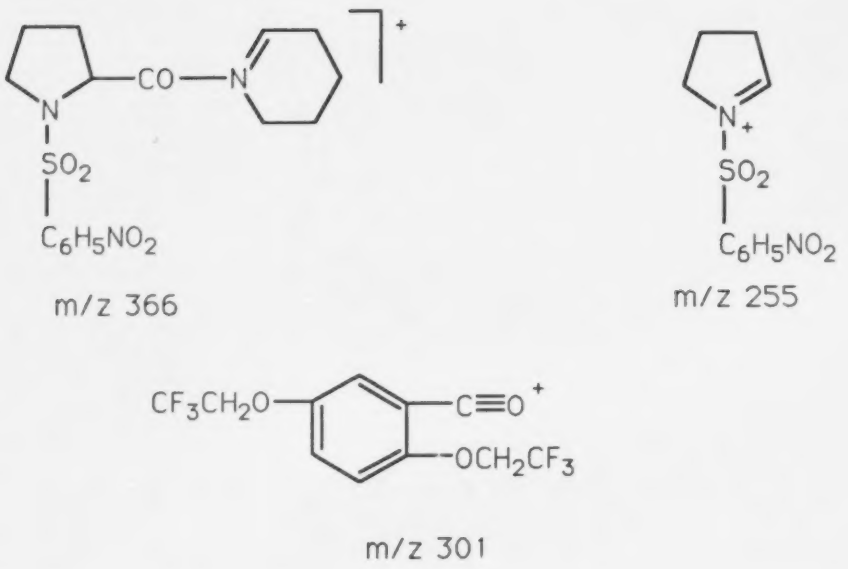

Figure 1.2.4. Proposed structures for diagnostic fragment ions in the mass spectrum of derivatized (R,S)-flecainide.

Figure 1.2.5. Chromatograms of (A) blank plasma spiked with 750 ng of racemic flecainide and internal standard; (B) plasma sample taken from a patient 5h after oral administration of a 100-mg dose of racemic flecainide; and (C) blank plasma. Peaks 1 and 2 are the internal standard diastereoisomers, and peaks 3 and 4 are the diastereomers of R- and S-flecainide, respectively.

observed between the peak area ratios (R- and S- flecainide:IS) and the corresponding plasma concentrations, in the range examined. Typical plasma standard curves gave the following equations : $y = 0.0093 + 0.0017x$ for R-flecainide and $y = 0.0129 + 0.0016x$ for S-flecainide with $r = 0.998$ (Figure 1.2.6.). Responses for the diastereomers were similar, as reflected by the slopes of the best-fit lines, and confirm that asymmetric induction does not occur under the experimental conditions.

Intraday precision and accuracy were satisfactory (Table 1.2.1). The interday variations were 3.9 and 9.6 for S-flecainide and 5.4 and 6.5 for R-flecainide using 250 and 500 ng/mL solutions, respectively (Table 1.2.2). The sensitivity of the method, as defined by the lowest quantifiable concentration with acceptable accuracy and precision, was set at 50 ng/mL for each enantiomer. With a 3:1 signal : noise ratio, the lowest limit of detection would be in the order of a few nanograms. The UV absorption maximum for flecainide diastereomers was 206 nm, but due to interference problems, 280 nm was selected as the analytical wavelength. Fluorescence detection proved to be inadequate since the natural fluorescence of flecainide was quenched following derivatization. Sample analysis by normal-phase HPLC provided run times approximately half of those observed with reverse-phase. Resolution was also superior ($\alpha=1.26$, $R_s=2.25$) but these chromatographic conditions were not pursued due to poor sensitivity.

In order to assess the applicability of the method to therapeutic monitoring and pharmacokinetic studies, the plasma level-time

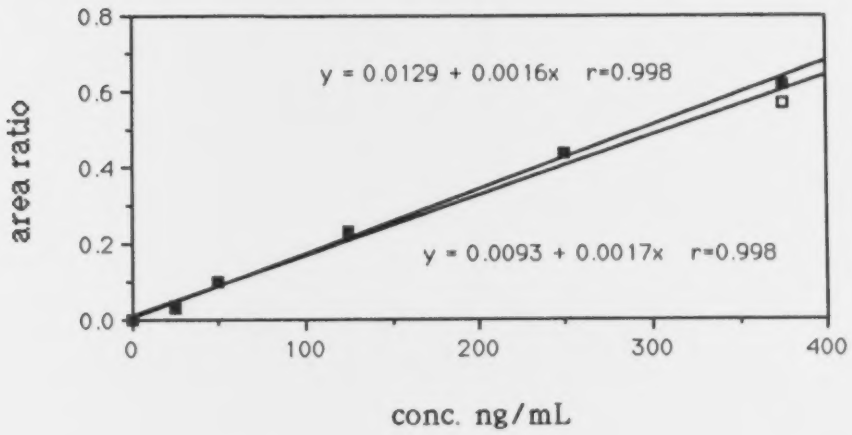

Figure 1.2.6. Calibration curves for S- (■) and R-flecainide (□). Five data points are superimposed.

Table 1.2.1. - Intraday Accuracy and Precision

Concentration, ng/mL						
Theoretical	Measured		Error, %		CV, %	
	R	S	R	S	R	S
500	492.2	480.8	-1.6	-3.8	3.7	2.8
375	354.2	355.2	-5.5	-5.3	5.6	1.1
250	235.9	234.3	-5.6	-6.3	8.5	9.8
125	122.1	116.2	-2.3	-7.0	3.6	9.2
50	44.4	42.7	-11.2	-14.6	4.9	4.4

n=4

Table 1.2.2.- Interday Accuracy and Precision

Concentration, ng/mL						
Theoretical	Measured		Error, %		CV, %	
	R	S	R	S	R	S
500	530.1	522.3	6.0	4.5	6.5	9.6
250	266.4	269.0	6.6	7.6	5.4	3.9

n=4

profiles of flecainide enantiomers were determined in a female patient. The steady-state plasma time courses are depicted in Figure 1.2.7. The plasma concentrations of the R enantiomer were slightly, but significantly, greater than those of S-flecainide, when a paired t-test was performed. The cumulative amounts excreted over 6-h time period were 15.1 and 19.6 mg for the R and S enantiomers, respectively. The clinical significance and the generality of this observation remain to be tested in a larger patient population.

The suggested therapeutic range for racemic flecainide is 200-1000 ng/mL although plasma levels of up to 1500 ng/mL appear to be tolerated [37,38]. However, as with other antiarrhythmic drugs, excessive flecainide concentrations are associated with considerable toxicity, including negative inotropic action and pro-arrhythmic effects [39]. Plasma level monitoring to guide dosage adjustment is recommended, particularly in patients with impaired renal [40,41] or hepatic [42] function. It has recently been suggested that genetic polymorphism plays a role in the metabolism of flecainide and that this may help explain the considerable variation in clearance and elimination half-life observed in patients [43].

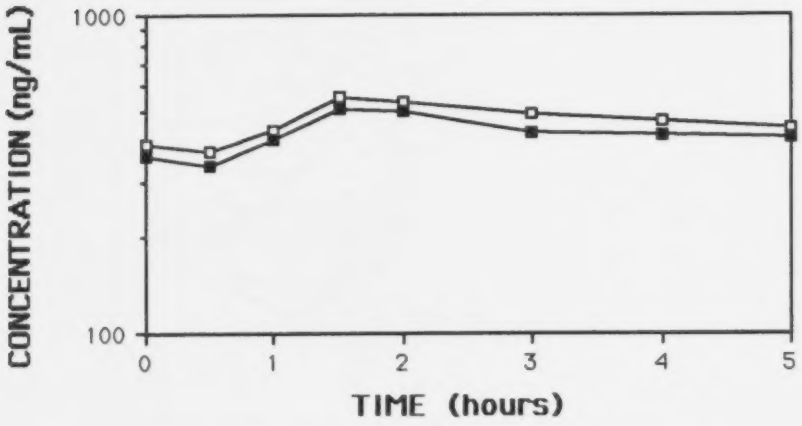

Figure 1.2.7. Steady-state plasma concentration-time profiles of R- (□) and S-flecainide (■) in one patient following repeated oral administration of a 100 mg/8h dose of racemic flecainide.

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1.3. HPLC analysis of flecainide enantiomers in human plasma: comparison with fluorescence polarization immunoassay*

At this point the purpose of developing a stereospecific assay for the determination of flecainide enantiomers in biological fluids, which was applicable to the study of pharmacokinetics in humans and to therapeutic drug monitoring, was achieved. The preliminary study of the plasma concentration-time curve in patients had shown an essentially parallel pattern for the two enantiomers. Since the pharmacological activities of R- and S-flecainide were already known to be similar, it was unlikely that clinical significance would derive from a more complete pharmacokinetic study. Attention was drawn to therapeutic monitoring of flecainide which was known to have a relatively narrow therapeutic index, and was routinely prescribed to several patients at the University of Alberta Hospital. Contact was made with the Director of the Toxicology Department who was interested in verifying the newly introduced methodology, FPIA (fluorescence polarization immunoassay), for the determination of flecainide plasma levels. Over a period of about two months plasma levels of patients in therapy with racemic flecainide were measured using the stereospecific HPLC method, and results were compared with the measurements obtained with FPIA. The outcome of this study is reported in the following section.

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* A version of this chapter has been published:
Alessi-Severini S, LeGatt DF , Pasutto FM, Jamali F, Coutts RT. *Clin Chem* 37 111-112 (1991). Correction *Clin Chem* 37 886 (1991).

(R,S)-Flecainide acetate is a racemic, class 1c antiarrhythmic agent used for the control of ventricular and supraventricular arrhythmias. In response to findings that flecainide has been associated with fatalities in clinical trials, the manufacturer now recommends that this drug be reserved for the treatment of life-threatening ventricular and junctional arrhythmias [1]. Concentrations of flecainide in plasma should be monitored to maintain the recommended therapeutic range, 0.2-1.0 mg/L.

Fluorescence polarization immunoassay (FPIA) has become the most popular technique for routinely processing multiple samples for drug analyses in a clinical setting because of the small sample volume required (50 μL) and high analytical throughput (18 samples/15 minutes) and because there is no need for sample pretreatment. A commercially available (Abbott Laboratories, Diagnostic Division, Irving, TX, USA) FPIA kit for determining (R,S)-flecainide acetate in plasma requires the use of the Abbott TDx system. However, this method is not stereospecific; data from such assays that do not discriminate between the enantiomers of a racemic drug may be misleading [2].

Recently an HPLC assay for flecainide enantiomers was reported [3]. Due to concerns about stability (pg. 13), all samples were analyzed immediately upon receipt. Plasma samples were alkalinized, extracted with diethyl ether and derivatized; the diastereomeric derivatives were separated on a μ-Bondapak C_{18} column, with detection at 280 nm. To determine the correlation between FPIA and stereospecific HPLC data, 16 standards and

plasma samples from 23 patients being treated with flecainide acetate were analysed by HPLC and FPIA.

The total concentrations of flecainide acetate (the sum of R- and S-flecainide) in plasma correlated significantly with the TDx measurements (Pearson's correlation coefficient $r=0.96$, $p<0.00001$). Results from the two methods were linearly related (slope = 1.00, intercept = 0.0084 μg/mL) (Figure 1.3.1), with no statistically significant difference (by Student's paired t-test) in the mean (± SD) concentrations of plasma samples: HPLC, 0.53 ± 0.25; TDx, 0.53 ± 0.26 μg/mL).

Results for standards formulated in water were 30% lower by TDx than by HPLC; these results were not included in the statistics. Matrix differences (pH, ionic strength, protein concentration) may affect the nature of antibody binding in the FPIA analytical procedure.

Experiments conducted on flecainide-supplemented plasma samples (n=36) containing different percentages of the two enantiomers showed no statistically significant difference between measurements by HPLC and those by FPIA when the R/S ratio was 0.1-9. However, the size of the error percent increased (TDx measurements were 20% lower than HPLC results and target values) when this R/S range was exceeded. Interestingly, the TDx measurements of plasma standards of known concentration of individual enantiomers were significantly and consistently lower than the corresponding HPLC results and target values (by ANOVA analysis followed by Duncan's Multiple Range Test). This observation raises some doubt as to the reliability of TDx for the analysis of

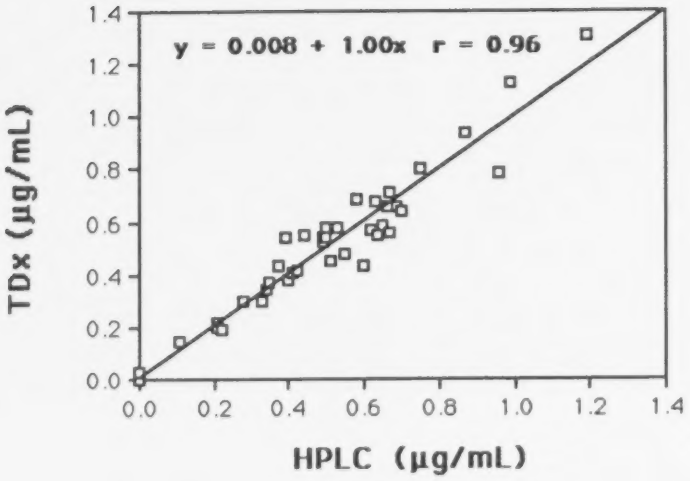

Figure 1.3. Correlation of TDx and HPLC concentration measurements in plasma.

individual enantiomers. However, flecainide acetate is administered as a racemate, and, because the enantiomers of flecainide have equivalent pharmacological properties [4,5], parallel pharmacokinetic patterns [3,6,7], and exhibit an R/S ratio in humans near unity (ranging between 0.75 and 1.33, mean 1.02±0.17) (Table 1.3.1), this discrepancy is unlikely to have clinical relevance. During the preparation of this manuscipt, a similar report [8] comparing a stereospecific HPLC assay and FPIA found excellent linearity between the concentrations of R- and S-flecainide and total flecainide values by FPIA. However, this did not prove that the corresponding values were superimposable. In our experience, even though an excellent linear relationship may be obtained between HPLC (or target values) and FPIA measurement of pure enantiomers, the slope of the curve (0.8) showed that FPIA results were consistently 20% lower than those by HPLC and target values.

Regarding the pharmacokinetics of flecainide enantiomers in humans, a recent report [7] describes the effect of sparteine-debrisoquine phenotype on the stereoselective elimination of this drug: pharmacokinetic patterns for the two enantiomers were absolutely superimposable in extensive metabolisers (R/S ratio=1) but displayed stereoselective metabolism in poor metabolizers, with R-flecainide predominanting in the plasma (R/S ratio >1). However, this study cannot be considered conclusive because it is not in agreement with three reports of stereoselective therapeutic monitoring [6, 8, 9], which found R/S ratio ranges of 0.75-1.33 (mean 1.02±0.17) (Table 1.3.1), 0.75-1.44 (mean 1.04) and 0.89-1.32 (mean 1.10±0.13), respectively. If R/S ratios >1 were to be genetically

TABLE 1.3.1. Enantiomeric ratios in patients.

SAMPLE NUMBER	R μg/mL	S μg/mL	Ratio R/S
1	0.20	0.24	0.83
2	0.26	0.27	0.96
3	0.43	0.33	1.30
4	0.28	0.32	0.88
5	0.27	0.31	0.87
6	0.45	0.51	0.88
7	0.17	0.15	1.13
8	0.34	0.30	1.13
9	0.34	0.28	1.21
10	0.24	0.27	0.89
11	0.18	0.23	0.78
12	0.34	0.35	0.97
13	0.20	0.19	1.05
14	0.18	0.19	0.95
15	0.26	0.24	1.08
16	0.33	0.32	1.03
17	0.49	0.50	0.98
18	0.12	0.09	1.33
19	0.11	0.10	1.10
20	0.34	0.33	1.03
21	0.28	0.21	1.33
22	0.15	0.20	0.75
23	0.39	0.36	1.08

controlled and to represent poor metabolizers of sparteine-debrisoquine, the incidence of this phenotype in the Caucasian population would have to be 10 %.

This was not evident in our study whose results combined with those from other reports [8,9] (for a total of 72 patients), show a frequency of 30% for R/S ratio >1. There is no evidence of bimodal distribution, and an equally high incidence of individuals showing R/S<1 remains unexplained on the basis of sparteine-debrisoquine polymorphism. Further investigation is required to clarify whether this discrepancy is attributable to different nongenetic factors affecting the patient population or whether other metabolic pathways have any relevance in the elimination of flecainide in patients with different metabolic impairments.

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1.4. Conclusion

Three stereospecific analytical methods were published in parallel with our work reported in chapters 1.2 and 1.3 [1-3]. The studies conducted in humans by other research groups were comparable to those reported herein and revealed that the pharmacokinetic patterns of the two enantiomers following oral administration in humans appear to be essentially parallel, and in some cases superimposable. Stereoselective elimination has been suggested for flecainide in healthy subjects classified as poor metabolizers of sparteine/debrisoquine, with R-flecainide always predominant in plasma after oral administration of the racemate. The oral AUCs of the enantiomers as well as the elimination half-lives were slightly, but significantly, different. Interpretation of this observation has been found in stereoselective hepatic metabolism [4]. The R/S ratio reported for different patient populations, nevertheless, seems to range from 0.75 to 1.44 [1, 5, 6], and the incidence of patients showing plasma levels higher in S-flecainide is much greater than the 5-10% reported for the poor metabolizers of debrisoquine. Other factors must therefore be of importance in the elimination of this drug such as genetic differences in metabolism affecting other pathways (mephenytoin polymorphism, environmental pollutants, diseases). The drug has been prescribed to a variety of patients affected by numerous different ailments that can modify the elimination of flecainide. The effect of these variables has not been taken into consideration.

The main characteristic of this drug is the great interindividual variability. Therapeutic plasma levels can be obtained with different levels of dosage and severe side effects can be observed in some patients at plasma levels that are usually well tolerated [7]. The pro-arrhythmic effects which produced high incidence of fatalities in the CAST investigation have not been explained on the basis of the present knowledge of the pharmacokinetics and pharmacodynamics of flecainide enantiomers. Since plasma levels of flecainide in patients that participated in the CAST were not published, it is impossible to speculate on the correlation between dose and toxic response. It seems unlikely that significant toxicity differences will be found between the enantiomers because the toxic effect is strictly correlated to the pharmacological effect of membrane depolarization, and this is similar for the two isomers. The reason why particular patients with asymptomatic ventricular arrhythmias following myocardial infarction are extremely vulnerable to the pro-arrhythmic effect of this drug remains obscure.

At this point it was felt that the initial goals proposed for this study had been achieved and that further investigation regarding the toxicity of this drug would involve approaches other than pharmacokinetic ones and would enter fields of investigation such as toxicology and clinical pharmacology. Furthermore, the fact that flecainide became restricted dramatically limited the number of patients available to us for any further study.

Racemic flecainide acetate was recently approved for oral use to prevent supraventricular arrhythmias, such as paroxysmal supraventricular tachycardias and paroxysmal atrial fibrillation [8].

It is still indicated for ventricular arrhythmias, but only in cases that in the judgement of the physician are life-threatening (e.g. sustained ventricular tachycardia). The maximum dose recommended is 400 mg a day [9].

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2. METHOCARBAMOL

2.1. Introduction*

Attention was directed towards the widely used muscle-relaxant methocarbamol whose chirality had been ignored for decades. It is surprising that there is limited information concerning the pharmacokinetics of this drug and that most of the general assumptions with respect to its activity and metabolism were published in the 1950s. In light of these considerations it was felt to be important to develop a stereospecific method for the determination of methocarbamol enantiomers in plasma and urine which could be applicable to pharmacokinetic investigation in humans and in animal models.

2.1.1. History

(R,S)-methocarbamol, 3-(2-methoxyphenoxy)-1,2-propanediol-1-carbamate, was developed in 1956 in the laboratories of A. H. Robins. Studies were directed towards the development of propanediol derivatives which possessed muscle relaxant properties superior to those of mephenesin, which had low potency and a short duration of action [1-3].

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2.1.2. Physicochemical properties

Molecular weight

$C_{11}H_{15}NO_5$= 241.24

Appearance, color and odor

Colorless (white) crystals or powder. Odorless or with a slight characteristic odor.

Optical rotation and absolute configuration

The optical rotation of R-methocarbamol (sodium D line, 1 dm cells, methanol as solvent) was obtained with a Perkin Elmer Model 241 polarimeter. With a 40 mg/mL solution the rotation was found to be (+)0.027; $[\alpha]^{26}_{D}$= (+) 0.68.

Based on the optical rotation value obtained under the conditions described above, the absolute configuration is R-(+)-methocarbamol.

Melting point

(R,S)-methocarbamol 90°-94° C [4].

S-methocarbamol 110-112° C.

R-methocarbamol 106-107° C.

Ionization constant

Methocarbamol is essentially a neutral compound. It can be extracted from aqueous acidic or alkaline media using organic solvents [4].

Distribution coefficient

The logP value has been found to be -0.06 [5].

Solubility

Methocarbamol is reported to be soluble in 1 in 40 of water [4] (2.5 g in 100 mL at 20° C) [6], soluble in propylene glycol, soluble in ethanol with heating, sparingly soluble in chloroform and practically insoluble in n-hexane [4,7].

2.1.3. Pharmacodynamics, pharmacokinetics and metabolism

Methocarbamol has been described as a centrally active muscle relaxant with the site of action identified as the spinal internuncial neuron [2,8]. A supraspinal locus of activity has also been suggested based on the protection provided against pentylenetetrazole- and electroshock-induced convulsions, and prolongation of the hexobarbital sleeping time [2]. However, direct evidence for a specific receptor has not been delineated and a complete understanding of the mechanism of action is not yet apparent [9]. The inhibition of carbonic anhydrases I and II by methocarbamol is not sufficiently strong to have implications in its mechanism of action [10]. A significant anxiolytic action for methocarbamol has been proposed recently [11]. Several early clinical studies (1958 and 1959) assessed the efficacy of methocarbamol in a variety of conditions including acute trauma and chronic neurological states [12], post-operative pain in gynecological patients [13], neuromuscular disease [14,15], and orthopedic situations [16].

Published pharmacokinetic information is comparatively scarce. Early studies in animal models (rat and dog), based on radioactive

tracer and colorimetric techniques, showed that methocarbamol was readily absorbed from the intestine and widely distributed in body fluids and tissues, particularly liver and kidney [17]. The reported bioavailability in animal models and in humans ranges from 36% and 90% [17-21]. Reduced absorption and first pass effects were proposed to explain the lowest values. Evaluations in the horse [22] and rat [19] revealed dose-dependent elimination on the basis of HPLC determination of plasma levels. The plasma half-life is reported to be between 0.5 and 1.6 h in animal models [18,19,22,23] whereas in human subjects the range is 1-2 h [20,24]. It appears that 98 % of the dose is eliminated in the urine, with <1% as the unchanged drug, and the remainder as free and conjugated metabolites. Most of the excretion seems to occur in the first 8 hours [4,17,21,23]. The major pathways of metabolism are O-demethylation and p-hydroxylation followed by conjugation with glucuronic acid or sulphate [4,17, 21,23]. Major species differences in the metabolism and disposition of methocarbamol have not been reported [17,23].

Methocarbamol is available by either prescription or as an over-the-counter product, often in combination with analgesics (acetylsalicylic acid, acetaminophen, codeine). It appears to be a relatively safe drug, one which is well-tolerated and widely used. Nevertheless, cases of fatal intoxication involving methocarbamol have been reported [25-27] and a potential for abuse has also been evaluated in individuals with a history of recreational substance abuse [28,29].

Methocarbamol has an asymmetric carbon and is administered as a racemate at significantly high doses (up to 8 g per day) [30]. It

appears that there has been no published information, during the three decades that methocarbamol has been on the market, on the pharmacodynamics and pharmacokinetics of methocarbamol enantiomers. After the development of a stereospecific assay of methocarbamol in biological fluids (reported in chapter 2.2), the pharmacokinetics of S- and R-methocarbamol was studied after administration of the racemate in the rat and in human healthy volunteers in the attempt to expand the knowledge on this drug. Details of this research are provided in chapters 2.3 and 2.4.

2.1.4. References

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2.2 High-performance liquid chromatographic analysis of methocarbamol enantiomers in biological fluids*

2.2.1. Introduction

(R,S)-Methocarbamol [(R,S)-3-(2-methoxyphenoxy)-1,2-propanediol-1-carbamate] is a well-known skeletal muscle relaxant which has been commercially available for over three decades. It is formulated as a single entity and, in combination with such analgesics as acetylsalicylic acid, acetaminophen and codeine, used for the relief of discomfort associated with acute, painful musculoskeletal conditions [1].

Human and animal studies show that about 98% of a dose is excreted in the urine in 72 h, with less than 1% as unchanged drug and the remainder as unconjugated and conjugated metabolites [2-5]. The two major metabolites isolated in humans and rats are inactive and have been identified as 3-(2-hydroxyphenoxy)-1,2-propanediol-1-carbamate and 3-(4-hydroxy-2-methoxyphenoxy)-1,2-propanediol-1-carbamate [2,3].

The literature contains only limited information on the pharmacokinetics of methocarbamol. The plasma half-life in humans ranges from 1 to 2 h [6,7] and is reported to be between 0.5 and 1.6 h in animal models (rat, dog and horse) [3,5,8]. There do not appear

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to be any major differences among species studied in the metabolism and disposition of methocarbamol [2]. Dose-dependent kinetics have been suggested in the rat [8] and the horse [5].

(R,S)-Methocarbamol is a chiral drug (Fig. 2.2.1) which is administered as a racemate. We are not aware of any published literature on the pharmacodynamic and pharmacokinetic properties of the enantiomers. In recent years it has been established that non-stereoselective data generated for racemic drugs can be misleading [9]. In light of these observations it is worthwhile to investigate methocarbamol enantiomers in order to obtain a complete and accurate understanding of the biological fate of this drug.

The specific purpose of this study was to develop a stereospecific assay which could be used to determine the pharmacokinetics of methocarbamol enantiomers in humans and in animal models.

2.2.2. Experimental Section

2.2.2.1. Chemicals and reagents

(R)-(-)-Flecainide (internal standard) was a gift from Riker Laboratories (St. Paul, MN), while (S)-(+)-1-(1-naphthyl)ethyl isocyanate (NEIC) and (R)-(-)-1-(1-naphthyl) ethyl isocyanate were purchased from Aldrich Chemical Company (Milwaukee, WI).

Racemic methocarbamol and R-methocarbamol were gifts from Whitehall-Robins Inc. (Mississauga, Canada) and Lee Laboratories Inc. (Petersburg, VA) respectively. Ethyl acetate was obtained from BDH Chemicals (Edmonton, Canada) and was glass distilled prior to use. Analytical grade hexane and isopropanol were also obtained

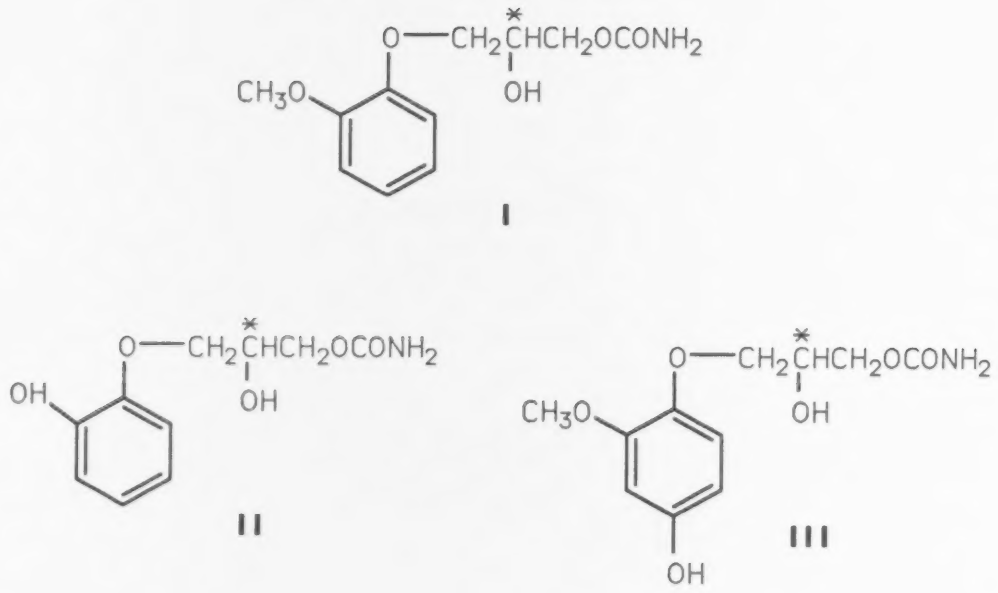

Figure 2.2.1. Structures of (R,S)-methocarbamol (I) and its major metabolites, 3-(2-hydroxyphenoxy)-1,2-propanediol-1-carbamate (II) and 3-(4-hydroxy-2-methoxyphenoxy)-1,2-propanediol-1-carbamate (III). The asterisk indidates a chiral centre.

from BDH Chemicals. HPLC grade water was purchased from Canlab (Edmonton, Canada). β-Glucuronidase/arylsulfatase EC 3.2.1.31 / EC 3.1.6.1 from *Helix pomatia* was purchased from Boehringer Mannheim (Laval, Canada).

A stock solution of (R,S)-methocarbamol was obtained by dissolving 10 mg in 100 mL of water. Working solutions of methocarbamol in plasma and urine were then prepared to give final concentrations of 0.5, 1, 2, 5, 10, and 30 µg/mL. A 10 µg/mL aqueous working solution of the internal standard was prepared by dissolving 1 mg of (R)-(-)-flecainide acetate in 100 mL. The 0.2% solution of (S)-(+)-1-(1-naphthyl)ethyl isocyanate in ethyl acetate was made by diluting 20 µl of reagent in 10 mL of solvent.

2.2.2.2. Instrumentation

The HPLC system (Waters Scientific, Mississauga, Canada) consisted of a 590 pump, a 481 variable wavelength UV detector set at 280 nm and a 3390A integrator-recorder (Hewlett-Packard, Mississauga, Canada). Electron-impact mass spectra were recorded on a double-focusing magnetic sector instrument (Kratos MS-50, Manchester, UK). Optical rotations of the derivatives as well as that of R-methocarbamol were measured in a Perkin-Elmer 241 polarimeter (Rexdale, Canada). UV spectra were recorded on a PU 8700 scanning UV/visible spectrophotometer (Philips, U.K.). NMR spectra were recorded on a high resolution Bruker AM 300 fourier transform spectrometer (Bruker Spectrospin, Milton, Canada) with tetramethylsilane as internal standard. Samples were mixed with an IKA Vibrax UXR mechanical shaker (Terochem, Edmonton, Canada),

centrifuged with a Clay-Adams centrifuge (Parsippany, New Jersey), and heated in a Reacti-therm block (Pierce Chemicals, Rockford, IL).

2.2.2.3. Chromatographic conditions

A Partisil 5 silica analytical column (250 x 4.6 mm i.d.) (Whatman, Clifton, NJ) was used for the chromatographic separation of the diastereoisomers. The mobile phase was a mixture of hexane and isopropanol (95:5 v/v) and the flow rate was 1.6 mL/min. A silica PrepNova-PAK HR column [6 µm, 100 x 25 mm PrepPak Cartridge] (Waters Scientific, Mississauga, Canada) was used for the semi-preparative separation of methocarbamol diastereomers. The reversed-phase analytical column µ−Bondapak C18 (5 µm, 300 x 3.9 mm i.d.) was purchased from Waters Scientific (Mississauga, Canada). The fused silica Chirasil-Val III gas chromatographic capillary column (50 m) was obtained from Terochem (Edmonton, Canada), by using a temperature program of 70°-230°C, rate 8°C/min, underivatized methocarbamol showed a retention time of approximately 8 min. The DB5 (50 m) and DB1 (50 m) columns were purchased from J &W Scientific (Rancho Cordova, CA). On the DB1 column (oven temperature 100°-280°C, rate 4°C/min) using a flame ionization detector, methocarbamol showed a retention time of approximately 12 min.

2.2.2.4. Sample collection

One rat was sacrificed (cardiac puncture) 1.5 h after i.v. administration of a 30 mg/kg dose of (R,S)-methocarbamol. Another rat underwent surgical cannulation of the jugular vein. A 120 mg/kg

dose of (R,S)-methocarbamol was administered intravenously and blood samples were collected from the catheter at 0, 15, 30, 60, 90, 120, and 180 min. In one human subject a blood sample was taken by venipuncture 3 h after a 1 g oral dose. The 6 h human urine output was collected and a 1:10 diluted sample was analysed directly according to the above procedure. A similar aliquot was incubated with β-glucuronidase/sulfatase at 37°C for 18 h and subsequently analysed. After centrifugation, plasma samples were stored at -20°C until ready for analysis.

2.2.2.5. Sample analysis

Following addition of 0.3 mL of internal standard solution, plasma and diluted urine (1:10 v/v in distilled water) samples were basified with 1 mL of 1M NaOH, and 5 mL of ethyl acetate was added. After mixing on the mechanical shaker (20 min), the samples were centrifuged at 1000 x g (5 min). The organic phase was separated and evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 1 mL of distilled ethyl acetate and 80 µL of the NEIC reagent solution was added to each tube. The mixtures were vortex-mixed for 5 s and heated at 85°C for 12 h. After derivatization the samples were evaporated and reconstituted in 0.2 mL of distilled ethyl acetate. Aliquots of 0.03-0.15 mL were injected into the HPLC apparatus.

2.2.2.6. Accuracy and precision

The accuracy and precision of the method were evaluated by analysing four replicates of spiked plasma samples at each of three

concentrations against a calibration curve. The accuracy was evaluated as error percent [(mean of measured - mean of added) / mean of added] x 100, while the precision was given by the inter-day and intra-day coefficients of variation. The inter-assay reproducibility was also evaluated by computing the mean ± SD of the slope for different calibration curves at four different days.

2.2.3. Results and Discussion

Methocarbamol is a relatively simple molecule which appeared to be readily amenable to gas chromatographic analysis. However, reactions with trifluoroacetic or pentafluoropropionic anhydrides, followed by chromatography on a Chirasil - Val III column, failed to resolve the enantiomers.

Efforts were then directed to the preparation of diastereomers by reaction with enantiopure derivatizing reagents including (-)-menthyl chloroformate, 1-[(4-nitrophenyl)sulfonyl]-L-prolyl chloride, (-)-α-chlorophenylacetyl chloride, and (R,R)-O,O-diacetyl tartaric acid anhydride. These reagents did not react with (R,S)-methocarbamol, either at room temperature or after heating. However, following reaction with (S)-(+)-1-(1-naphthyl)ethyl isocyanate, the peak representing underivatized methocarbamol disappeared from the gas chromatographic trace; while derivatized methocarbamol was not observed under GC conditions, underivatized methocarbamol was clearly evident as a chromatographic peak having a retention time of approximately 12 min (see Experimental

for details). Unfortunately, chromatographic peaks corresponding to the diastereomers were not observed on this trace presumably as a consequence of thermal instability (numerous unidentified peaks were evident in the chromatograms). Gas chromatography was nevertheless useful during method development since the derivatization reactions could be optimized by monitoring for the disappearance of underivatized methocarbamol.

The reaction with NEIC does not occur to any significant extent under ambient conditions and while higher temperatures (up to 110°C) could reduce the reaction time to about 4-6 h, the increased proportion of interfering by-products (as evident by HPLC) was unacceptable. The reaction (Fig. 2.2.2) was considered complete when the peak of (R,S)-methocarbamol disappeared from the GC trace; this was accomplished after heating for 12 h at 85 °C. Efforts to facilitate the reaction by means of microwave irradiation were unsuccessful. Methocarbamol enantiomers were not resolved under reverse-phase LC conditions but were separated by normal phase with satisfactory results (α=1.24, R_S=2.10). This allowed sufficient discrimination of an unidentified interfering peak from the first eluting diastereomer (Fig.2.2.3). The diastereomers of methocarbamol eluted at 33 and 41 min (Fig.2.2.3) while the internal standard [(R)-(-)-flecainide] derivative eluted at 12 min. Guaifenesin, a possible product of methocarbamol decomposition, does not interfere with the assay as it was found to elute, after derivatization, at approximately 20 min under the experimental conditions.

For the purpose of structure elucidation, the diastereomers were isolated from the appropriate LC eluent fractions. Samples submitted

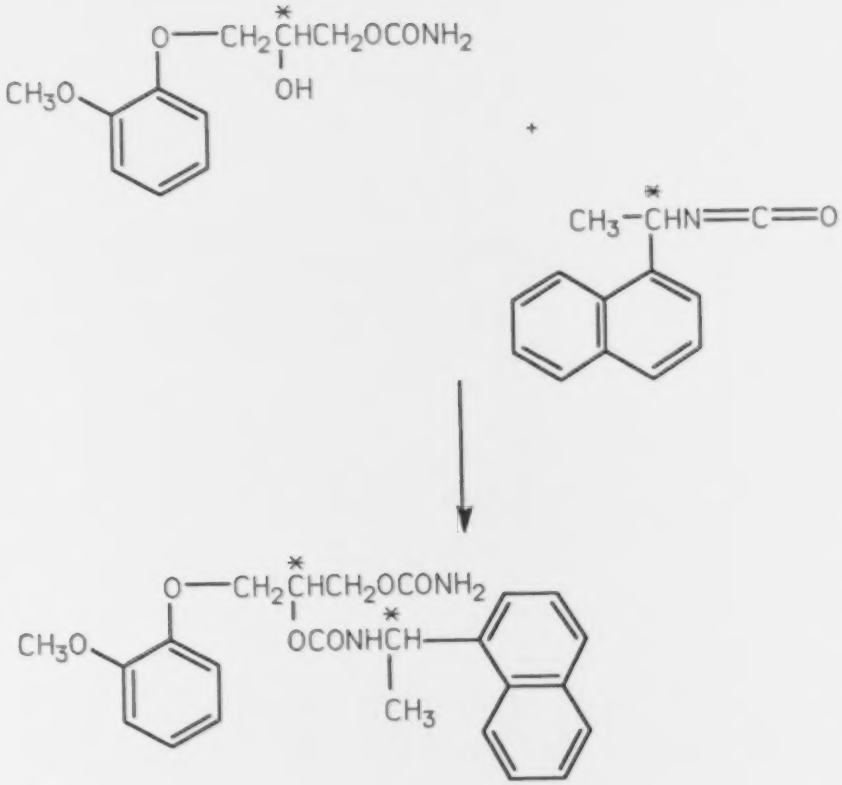

Figure 2.2.2. Derivatization reaction of (R,S)-methocarbamol with (S)-(+)-1-(1-naphthyl)ethyl isocyanate.

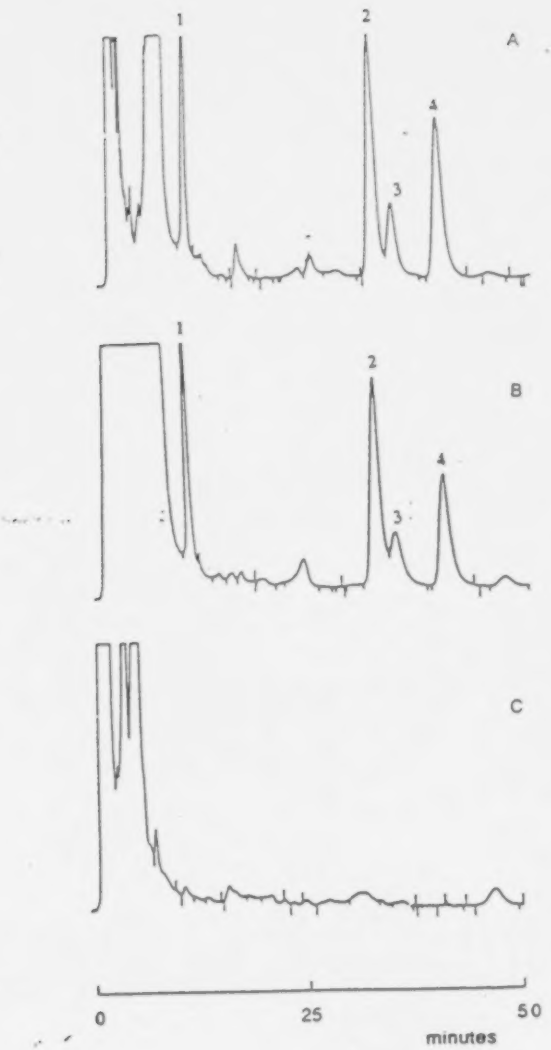

Figure 2.2.3. Chromatograms of (A) blank rat plasma spiked with 5 μg of racemic methocarbamol and internal standard; (B) plasma sample taken from a rat 1.5h after i.v. administration of a 30 mg/kg dose of racemic methocarbamol; and (C) blank rat plasma. Peak 1 is the derivatized (R)-flecainide; peaks 2 and 4 are the diastereomers of S - and R-methocarbamol, respectively; peak 3 is unidentified.

for mass spectral analyses were obtained by combining several fractions eluting from the analytical column, while optical rotation measurements and NMR analyses were determined following isolation of the respective diastereomers on the preparative LC system. The direct inlet EI-mass spectra of the corresponding diastereomer fractions were superimposable. While they lacked the molecular ion (m/z 438), they contained fragments of diagnostic value which indicated that derivatization takes place on the alcohol OH. Plausible fragments are suggested in Fig.2.2.4. NMR spectra also confirmed the structures of the two derivatives.

The UV spectra of the diastereomers were superimposable and showed absorption maxima at 206 and 280 nm. The latter was used as the HPLC analytical wavelength.

The optical rotations of the diastereomers were determined by polarimetry (sodium D line, 589 nm). The first eluting derivative was levorotatory while the second eluting derivative was dextrorotatory. A sample of pure (R)-(+)-methocarbamol in methanol examined under the same conditions gave a positive optical rotation.

The (+)-diastereomer that elutes at 41 min under the described chromatographic conditions corresponds to (R)-(+)-methocarbamol as confirmed by derivatizing and injecting a sample of pure (R)-methocarbamol. By exclusion the (-)-derivative was considered derived from (S)-(-)-methocarbamol. Reaction of the racemate and the pure (R)-(+)-methocarbamol with (R)-(-)-1-(1-naphthyl)ethyl isocyanate was equally successful and showed the expected transposition of retention times of the derivatives.

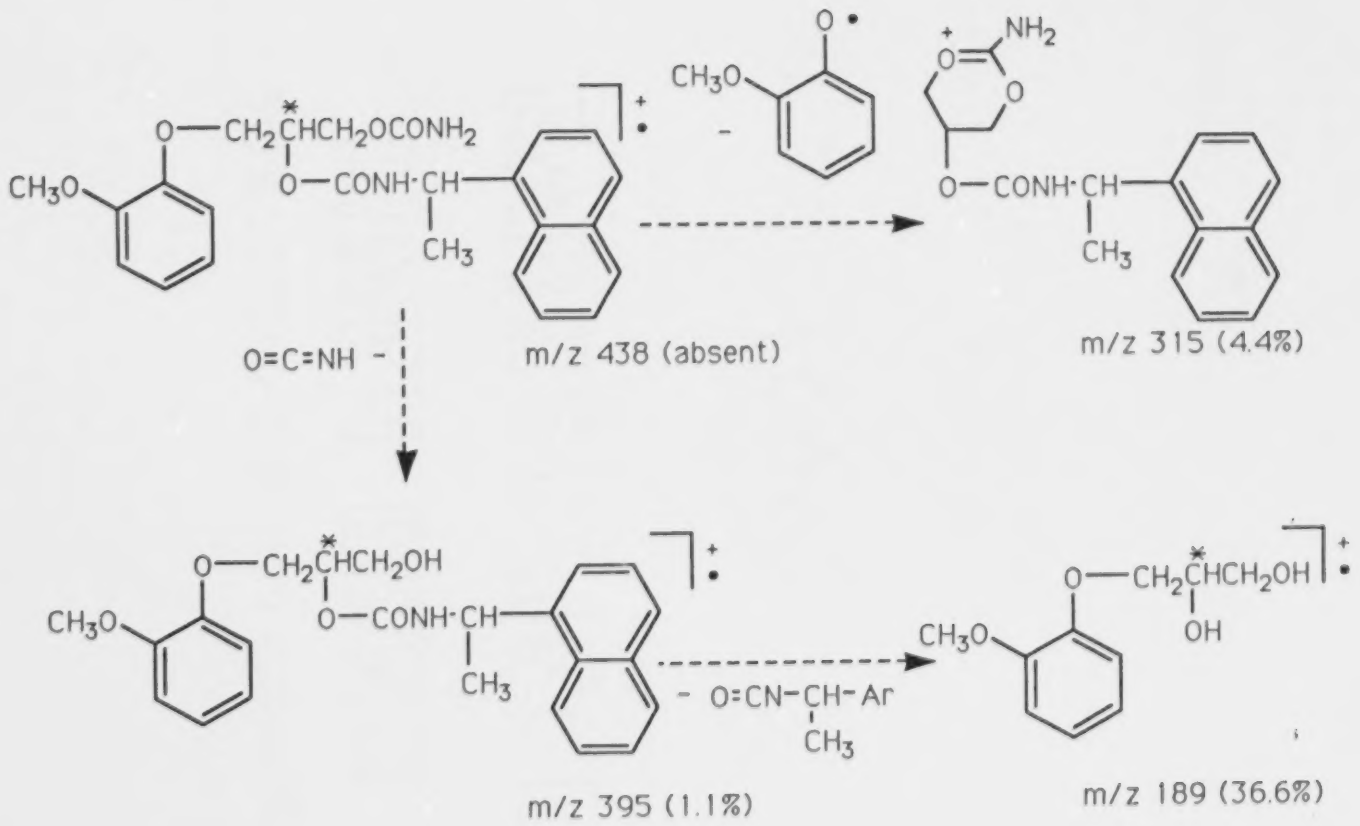

Figure 2.2.4. - EI-MS stuctures and proposed fragmentation mechanisms of the most diagnostic fragments of methocarbamol derivatives.

The extraction of methocarbamol isomers and (R)-(-)-flecainide was performed under alkaline conditions in order to eliminate possible interferences from phenolic metabolites and endogenous plasma constituents, and to facilitate a complete extraction of the basic internal standard. Ethyl acetate was preferred to diethyl ether, hexane, or chloroform as extraction solvent because it gave a better recovery of methocarbamol. Samples of 1 µg/mL and 10 µg/mL were analysed in duplicate and compared to samples derivatized directly without extraction. The amount of methocarbamol recovered was 0.93 and 0.94 µg/mL (mean 93.5%) at the low concentration, and 9.0 and 8.7 (mean 89.5%) at the high concentration.

Excellent linearity was observed between peak area ratios [methocarbamol derivatives:internal standard derivative] and plasma concentrations (range evaluated : 1-30 µg/mL of racemate). Typical calibration curves gave the following equations: $y = -0.05 + 0.86x$ for (S)-methocarbamol and $y = -0.10 + 0.99x$ for (R)-methocarbamol with $r=0.997$ and 0.999 respectively (Fig.2.2.5). Intraday precision and accuracy (Table 2.2.1) were satisfactory as were the interday precision and accuracy (Table 2.2.2). Values of the calibration curves slopes did not change significantly when evaluated on four different days, the means±SD were 0.83 ± 0.05 and 1.03 ± 0.03 for S- and R-methocarbamol respectively. The lowest quantifiable concentration was set at 0.5 µg/mL for each enantiomer. If a 3:1 signal/noise ratio criterion is applied, a detection limit of approximately 10 ng/mL could be claimed.

In order to assess the applicability of this analytical method to pharmacokinetic studies, plasma levels of methocarbamol

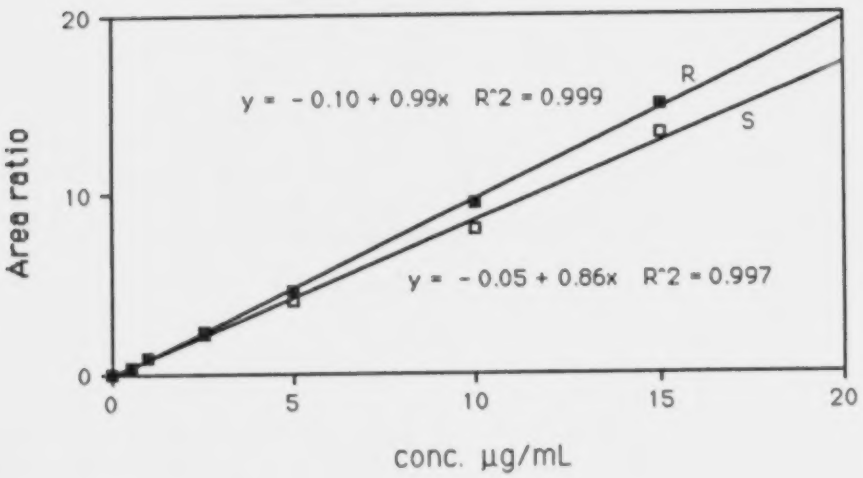

Figure 2.2.5. Calibration curves for S- (□) and R-methocarbamol (■).

Table 2.2.1.- Intraday Accuracy and Precision

Concentration, µg/mL			Error, %		CV, %	
Theoretical	Measured					
	(S)	(R)	(S)	(R)	(S)	(R)
5.00	5.50	5.50	10	10	0.9	0.9
1.00	0.96	0.89	-4	-11	2.1	1.1
0.50	0.52	0.51	4	2	3.8	3.9

n=4

Table 2.2.2.- Interday Accuracy and Precision

Concentration, µg/mL			Error, %		CV, %	
Theoretical	Measured					
	(S)	(R)	(S)	(R)	(S)	(R)
5.00	5.10	5.10	2	2	3.9	5.9
0.50	0.51	0.52	2	4	9.8	8.8

n=4

enantiomers were measured in one rat at 1.5 h after i.v. administration of a 30 mg/kg dose of racemic methocarbamol. The concentration of (S)-methocarbamol was 2.38 μg/mL whereas the concentration of (R)-methocarbamol was 0.99 μg/mL (S/R enantiomer ratio of 2.40). A plasma sample taken from a human volunteer 3 h after a 1 g oral dose contained 2.57 and 2.16 μg/mL of S- and R- enantiomer, respectively (S/R 1.19). A 6-h urine specimen contained 12.8 and 11.2 μg/mL (total output of 3.61 and 3.16 mg) (S/R 1.14). An aliquot of the 6-h urine specimen was analysed following incubation with β-glucuronidase/sulfatase at 37°C for 18 h. In this case the total output was 62.6 mg for (S)-methocarbamol and 51.3 mg for (R)-methocarbamol (S/R 1.22). The recovery of the (R,S)-methocarbamol initial dose excreted in 6 h as unchanged drug was therefore 0.68 % while the total drug (unchanged + conjugated) was 11.4 %. All these single point data are comparable with previously reported non-stereoselective data [2,3,6,7].

Plasma time-courses of methocarbamol enantiomers in one rat after i.v. administration of a 120 mg/kg dose of racemic methocarbamol are depicted in Fig. 2.2.6. Plasma concentrations between the two enantiomers showed a statistically significant difference when compared using a paired t-test.

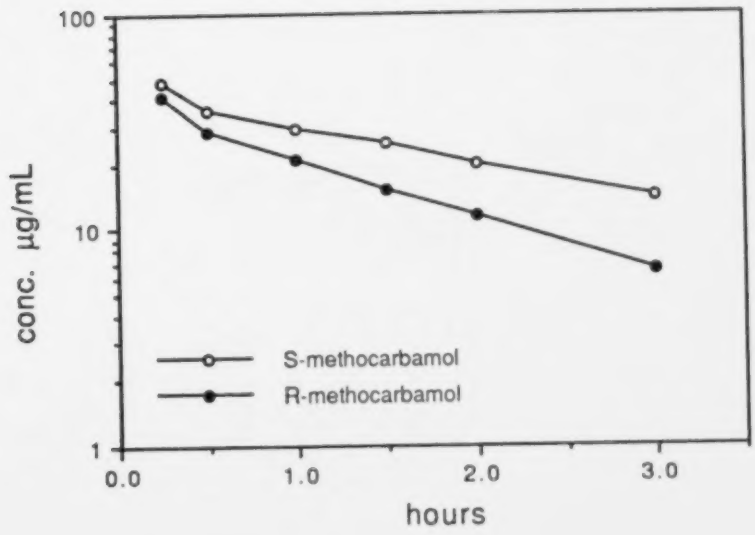

Figure 2.2.6. Plasma concentration-time profiles of methocarbamol enantiomers in one rat following a single intravenous dose of 120 mg/kg racemic methocarbamol.

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2.3. Pharmacokinetics of Methocarbamol Enantiomers in the Rat

2.3.1. Introduction

(R,S)-Methocarbamol is a centrally active muscle relaxant which is widely used for the control of skeletal muscle spasms. It was introduced as an improvement over mephenesin, mephenesin carbamate and guaifenesin because it showed a more prolonged action as a consequence of higher plasma levels in animal models [1] as well as in humans [2]. The metabolism of (R,S)-methocarbamol has been studied in humans [3,4], the rat [4], and the dog [3,4]; substantive species differences have not been observed. The major metabolic pathways include dealkylation, hydroxylation and conjugation with glucuronide and sulfate [5]. The two major metabolites in humans and in animal models are 3-(2-hydroxyphenoxy)-1,2-propanediol-1-carbamate and 3-(4-hydroxy-2-methoxyphenoxy)-1,2-propanediol-1-carbamate. It appears that only 1-8% of a dose is eliminated in the urine as unchanged methocarbamol; the rest is represented by metabolites, conjugated methocarbamol and conjugated metabolites [3,4,5]. There is only limited pharmacokinetic information available in the literature. Plasma clearance appears to decrease with increasing dose in the rat [6] and horse [7], suggesting dose-dependent elimination. Plasma half-life is 0.5 - 1.6 h [6,7,8] in animal models and 1-2 h in humans [9,10]. Bioavailabilities of 29 to 90 % have been reported [3,6,8,9,]

Methocarbamol is administered as the racemate; and to date there is no published literature on the pharmacodynamics or pharmacokinetics of the enantiomers. It seems, however, that S- and R-methocarbamol exhibit the same muscle relaxant potencies when tested against the racemate in the mouse traction test [11].

The purpose of this study was to investigate stereoselectivity in methocarbamol pharmacokinetics using a suitable animal model.

2.3.2. Experimental Section

2.3.2.1. Materials

Racemic methocarbamol was kindly supplied by Whitehall-Robins Inc. (Mississauga, Ontario, Canada).

2.3.2.2. Assay

Plasma and urine concentrations of S- and R-methocarbamol were determined using our recently reported HPLC assay [12]. Briefly, the method consisted of a one-step ethyl acetate extraction of the alkalinized sample, derivatization with (S)-(+)-1-(1-naphthyl)ethyl isocyanate and normal phase chromatography with UV detection at 280 nm. During the study the method was validated by checking the linearity of the calibration curves (coefficients of correlation ranged from 0.96 to 1.0) and analysing, at random, standards and blind samples that confirmed the quality of the quantification. In few instances, standard samples were also analysed in parallel with a non-stereospecific assay [6] in order to validate further the new methodology.

2.3.2.3. Procedure

A total of 24 female Sprague-Dawley rats (200-300g) were divided into 6 groups (n=3-6). Each of four groups received one of four doses (30, 60 120, or 220 mg/kg) of racemic methocarbamol through the jugular vein. A fifth group received 120 mg/kg of racemic methocarbamol solution by gastric gavage. The last group received the drug intraperitoneally at a dose of 120 mg/kg.

Rats were anesthetized with methoxyflurane (Metofane, Pitman-Moore, Mississauga, Ontario, Canada) prior to, and during surgery. The jugular vein was cannulated with polyethylene tubing (PE-50, Clay-Adams, Parsipanny, NJ, USA) tipped with Silastic tubing (0.025 inch i.d. x 0.047 inch o.d.; Dow-Corning, Midland, MI, USA). Animals were allowed at least an overnight recovery before drug administration. Racemic methocarbamol was dissolved in a 1:1 polyethylene glycol 400 / water mixture for administration by all three routes. Blood samples (0.20 mL) were drawn from the jugular vein cannula at 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6 h. The cannula was flushed with heparin (100 IU/mL) as required. The specimens were centrifuged immediately (Microcentrifuge model 235A, Fisher, Edmonton, Alberta, Canada) for 3 min and plasma was collected. All specimens were stored at -20°C before analysis. Urine was collected for up to 6 h after administration and was analysed for the total recovery of S- and R-methocarbamol.

In the protein binding study, 1 mL samples of blank rat plasma underwent equilibrium dialysis against 1 mL samples of isotonic phosphate buffer (pH 7.4) spiked to obtain final concentrations of 10 and 200 mg/L of racemic methocarbamol. The apparatus consisted of

Spectrum (Los Angeles, CA, USA) Teflon cells equipped with a Sigma Diagnostic (St. Louis, MO, USA) dialysis membrane. The equilibration experiments were conducted at 37°C for 4 h.

2.3.2.4. Data analysis

Areas under the plasma concentration-time curves (AUC) were calculated by the trapezoidal rule and extrapolated to infinity by dividing the last measured concentration by the overall elimination rate constant (β). Systemic clearance (CL_S) was calculated using the equation CL = dose / $AUC_{(0-\infty)}$. Half-life ($t_{1/2}$) was estimated using linear regression of the terminal log-linear phase. An estimate of oral bioavailability was determined by the ratio $AUC_{oral\ (0-4h)}/AUC_{iv(0-4h)}$. Vd_{ss} was determined by the ratio $D[AUMC]/[AUC]^2$.

To evaluate differences among pharmacokinetic parameters obtained at different levels of iv dosage, and for the three different routes of administration, one-way ANOVA analysis, when the F-test showed a significant difference among means, the Fisher(LDS) test and the Scheffe's F-test were performed in order to evidenciate which means were different. The paired Student's t-test was used to evaluate the significance of the differences between enantiomers. The level of significance was set at $p<0.05$.

2.3.3. Results

2.3.3.1. Intravenous pharmacokinetics

The pharmacokinetic parameters of S- and R-methocarbamol after i.v. administration of 30, 60, 120, and 220 mg/kg doses of the racemate are reported in Table 2.3.1. Data are characterized by broad inter-animal variation although definite trends can be observed. S-methocarbamol was generally predominant in plasma at all levels of dosage, in particular the S-methocarbamol AUC was significantly higher than that of R-methocarbamol at a dose of 120 mg/kg. S-methocarbamol plasma clearance was lower than that of the R-enantiomer and at the doses 60, 120 and 220 mg/kg it was significantly lower. The pharmacokinetics can be described as linear for both enantiomers in the dosage range of 30-220 mg/kg. Both enantiomers showed a trend toward decreased plasma clearance at the highest level of dosage in parallel with an increase in terminal half-life but no statistical significance was observed. Vd_{ss} did not show statistically significant differences between enantiomers and between doses. The proportionality of AUC increase with increasing dose is depicted in Figure 2.3.1.

2.3.3.3. Oral pharmacokinetics

The variability of the oral data in the terminal phase made it impossible to obtain a good linear regression for calculation of $AUCs_{(0-\infty)}$. Therefore AUCs were evaluated from 0 to 4 hours. The $AUCs_{(0-4h)}$, C_{max} and t_{max} for S-methocarbamol and R-methocarbamol, after administration of a 120 mg/kg dose, are

Table 2.3.1- Intravenous pharmacokinetics of methocarbamol enantiomers in the rat.

	AUC, mg L^{-1} h		CL_s, L kg^{-1} h^{-1}		$t_{1/2}$, h		Vd_{ss}, L kg^{-1}	
dose,mg/kg	S	R	S	R	S	R	S	R
30[1]	31.2 ± 8.6	24.0 ± 5.8	0.51 ± 0.13	0.66 ± 0.18	1.0±0.5	0.8±0.5	0.6±0.3	0.5±0.3
60[2]	57.7 ± 12.1	37.6 ± 3.5	0.53 ± 0.12	0.80 ± 0.08*	1.5±0.5	0.9±0.7	1.3±0.5	1.1±0.7
120[3]	140.2 ± 33.7	102.3 ± 20.3*	0.50 ± 0.14	0.61± 0.15*	1.6±1.2	0.9±0.3	1.3±0.9	0.9±0.3
220[4]	274.5 ± 51.5	201.3 ± 61.6	0.41 ± 0.08	0.59 ± 0.18*	2.5±1.1	1.4±1.0	1.6±1.2	1.6±0.9

[1] n=3
[2] n=3
[3] n=6
[4] n=4
* $p<0.05$ statistically different from the corresponding value for S-methocarbamol. Values are expressed as means±SD.

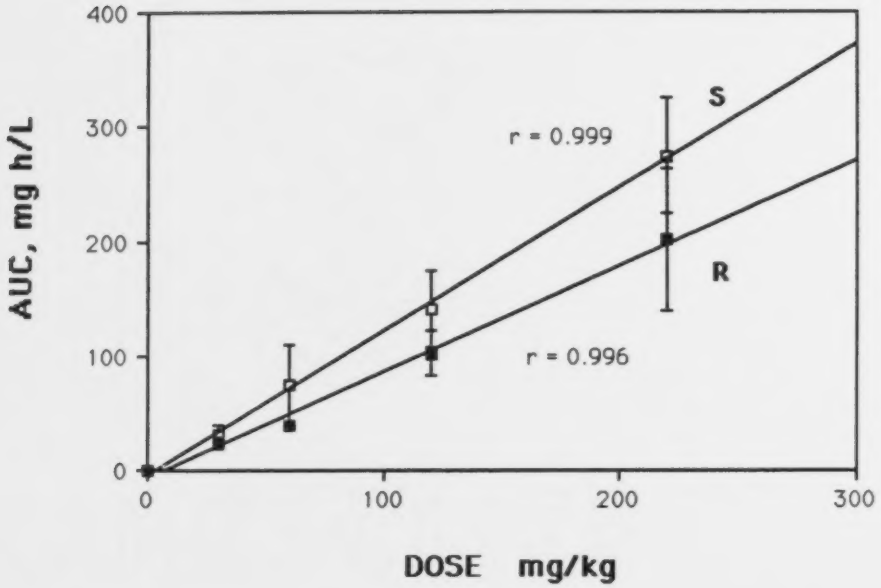

Figure 2.3.1. Dose vs AUC. S-methocarbamol (□) and R-methocarbamol (■). Values are expressed as means ± SD, n=3-6.

reported in Table 2.3.2. AUC and C_{max} values for the two enantiomers were not significantly different. Interestingly, t_{max} values were significantly different for the two enantiomers: 1.2 ± 0.3 h and 0.6 ± 0.4 h for S- and R-methocarbamol, respectively.

An estimation of bioavailability was calculated by dividing the mean $AUC_{po(0-4h)}$ by the mean $AUC_{iv(0-4h)}$. S-methocarbamol showed a mean F value of 0.32, whereas the mean F for R-methocarbamol was 0.35.

2.3.3.4. Intraperitoneal pharmacokinetics

As reported in Table 2.3.2, higher plasma levels for S-methocarbamol, compared to R-methocarbamol, were also observed with this route of administration. S-Methocarbamol AUCs were significantly higher than those of R-methocarbamol. Mean C_{max} values were also significantly different whereas, mean t_{max} values were comparable for the two enantiomers.

2.3.3.5. Urinary data

The percentage of the dose excreted in the urine as unchanged S- and R-methocarbamol did not differ significantly between enantiomers and iv doses (30 mg/kg: S-methocarbamol 2.2% R-methocarbamol 1.2%[1]; 60 mg/kg: S-methocarbamol 2.7%, R-methocarbamol 2.9 %[1]; 120 mg/kg: S-methocarbamol 3.8±1.8%, R-methocarbamol 3.5±2.0%; 220 mg/kg: S- 3.2±1.9%, R-methocarbamol 3.0±1.6%). Similarly, significant differences were not observed in

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1 only one determination was available for this group.

Table 2.3.2.- Pharmacokinetic parameters after oral, intraperitoneal, and intravenous administration of 120 mg/kg of racemic methocarbamol in the rat.

	p.o[1].		i.p[2].		i.v[3].	
	S	R	S	R	S	R
AUC (mg h/L)	36.9±19.3^	32.6±17.5^	123.4±36.7	71.7±22.0*	114.0±30.0	93.3±19.5*
C_{max} (mg/L)	15.8±8.0	16.2±10.8	64.1±21.7	36.8±9.7*		
t_{max} (h)	1.2±0.3	0.6±0.4*	0.88±0.2	0.88±0.2		

[1] n=4
[2] n=4
[3] n=6
^ $p<0.05$ (ANOVA and Scheffe's F-test) significantly different from the corresponding value after ip and iv administration.
* $p<0.05$ (Student's t-test) significantly lower than the corresponding value for S-methocarbamol. Values are expressed as means±SD.

urinary excretion of the unchanged methocarbamol enantiomers following oral (S-methocarbamol: 1.1±0.4%; R-methocarbamol: 0.8±0.3%) and intraperitoneal administration (S-methocarbamol: 1.4±0.4 %; R-methocarbamol 1.0±0.4%).

2.3.3.6. Protein binding

Plasma protein binding was evaluated at two different concentrations, 10 µg/mL and 200 µg/mL. S- and R-methocarbamol plasma protein binding was in the range of 42-44% and 46-58% respectively at the concentration of 10 µg/mL (n=5). At the higher concentration the ranges were 21-47% and 21-45 % respectively (n=5). One possible reason for the variability is the fact that the values were uncorrected for volume shifts. Stereoselectivity was not observed (S/R ratios were 0.9-1.3 and 1.0-1.1 respectively).

2.3.4. Discussion

Earlier studies in animals [3,4] and humans [4], based on radioactive tracer techniques and colorimetric determinations, showed that (R,S)-methocarbamol was readily absorbed from the gastrointestinal tract and widely distributed to tissues, particularly liver and kidney [3]. More recently, a non-stereospecific HPLC method was used to show that the bioavailability in rat ranged from 70 to 83 % [6]. Dose-dependency was proposed and a slight first-pass effect suggested. The site of first-pass metabolism was, however, not determined [6]. Another non-stereoselective HPLC method [8] was

used to evaluate bioavailability in the thoroughbred race horse: the mean value was 36.3%, with a range of 29-41% [8].

Since it is well recognised that non-stereospecific pharmacokinetic data for racemic drugs can be misleading, it was felt important to delineate the pharmacokinetics of S- and R-methocarbamol. The levels of dosage were chosen to reproduce plasma levels which have been reported in humans [9,10,12].

Results from iv administration showed that the pharmacokinetics of both enantiomers were linear in the range of doses tested (30-220 mg/kg) (Figure 2.3.1). A trend toward lower systemic clearances and higher half-life values at the 220 mg/kg dose was observed for both enantiomers, but was not supported by statistical significance in either case. The investigation of dose-dependency was not pursued further because plasma levels obtained in the rat, with an administration of 220 mg/kg, already exceeded the expected therapeutic values in humans. Since the clearances of methocarbamol enantiomers were much lower than the hepatic blood flow in the rat (reported to be 3.54 L $kg^{-1}h^{-1}$ [13]), the liver did not appear to be the most probable site of first-pass effect. However, the plasma levels obtained after oral administration of the drug clearly showed reductions in the bioavailabilities of the enantiomers. This suggested the possibility of impaired absorption and/or presystemic metabolism (other than hepatic). A comparison of AUCs, obtained after intravenous, oral and intraperitoneal administration of the same dose of racemic methocarbamol, was required in order to determine the most probable site of the first-pass effect.

The intraperitoneal route of administration delivers the drug into the hepatic portal vein, thus eliminating the possible contribution of first-pass in the gut. The dose of 120 mg/kg was chosen for comparative purposes since the plasma levels obtained in the rat were similar to those that produce therapeutic effects in humans. The mean oral $AUC_{(0-4h)}$ was significantly lower (approximately 35%) than the i.v. $AUC_{(0-4h)}$ for both enantiomers, indicating that the drug undergoes, in fact, a significant first-pass effect and that the oral bioavailability is in the order of 35% (mean F= 0.32 and 0.35 for S- and R-methocarbamol respectively). The difference between the mean i.v. $AUC_{(0-4h)}$ and mean i.p. $AUC_{(0-4h)}$ was not significant for either enantiomer, suggesting that the primary site of the first-pass effect is the gut. The possibility of reduced absorption was ruled out because an earlier study in the rat showed 98% recovery of an oral dose of labeled methocarbamol [4]. After intraperitoneal and intravenous administration, the S-methocarbamol AUCs were significantly higher than those of R-methocarbamol. However, after oral administration the statistical significance was lost and the two AUCs became similar; similarly, S-methocarbamol C_{max} was significantly higher than that of R-methocarbamol, after intraperitoneal administration, while after oral administration the two values became comparable. This observation may indicate a more pronounced gut metabolism for S-methocarbamol, however a conclusion on changes in stereoselectivity with route of administration cannot be drawn because the loss of statistical significance can be attributed to the larger variability observed in the oral data.

It was interesting to observe that t_{max} for S-methocarbamol was significantly higher (1.2 ± 0.3 h) than the corresponding value of 0.6 ± 0.4 h for R-methocarbamol, suggesting a delay in absorption of S-methocarbamol. Typical plasma concentration-time profiles for S- and R-methocarbamol after intravenous, intraperitoneal, and oral administration are depicted in Figure 2.3.2. These results should beconsidered preliminary with respect to bioavailability because the study was not controlled in terms of animal size, fasting and drug administration after surgery. There are some data to suggest that surgery has an effect on bioavailability [14]. Moreover, the sensitivity of the analytical method did not allow an accurate estimation of the terminal phase, especially after the oral administration. The experiment was carried out on a limited population of rats, therefore further studies are necessary to evaluate the generality of these observations. Nevertheless the suggestion of a low bioavailability (36%) for methocarbamol is substantiated by a recent report on the pharmacokinetics of methocarbamol in the horse [8]. It is also in agreement with indirect observations in an earlier report which showed that methocarbamol was three times less effective in inducing loss of the righting reflex in mice, rats, and hamsters after oral administration than after intraperitoneal administration [15]. Furthermore the recommended oral dose in humans [16] and animals (horses) [8] is 2-3 times as high as the intravenous dose. Controlled studies of absolute bioavailability in humans have not been reported.

Urinary excretion of unchanged enantiomers appears to be a minor pathway of elimination (lower than 7% of the dose for either

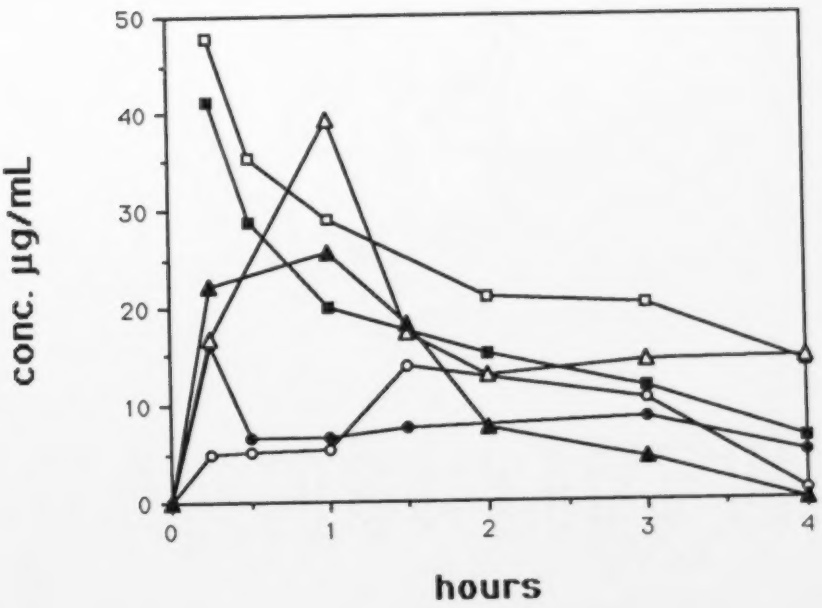

Figure 2.3.2. Plasma concentration-time profiles after i.v. (□ S-methocarbamol, ■ R-methocarbamol), oral (o S-methocarbamol, ● R-methocarbamol), and i.p. (Δ S-methocarbamol, ▲ R-methocarbamol) administration of a 120 mg/kg dose of the racemate.

enantiomer). Stereoselectivity was not apparent. The lower recovery after ip administration is explained by the fact that in those instances urine was collected only for 4 h, therefore no comparison was possible between ip and oral data nor between ip and iv data. However, it was interesting to note that the lower urinary recovery of both enantiomers after oral administration (6h-collection) compared to the iv administration (6h-collection), did show statistical significance ($p < 0.05$). The urinary excretion of unchangedenantiomers remains, nevertheless, a minor pathway in the elimination of methocarbamol, and for this reason it was not investigated further.

In conclusion, the pharmocokinetics of (R,S)-methocarbamol, as for all drugs eliminated by many different metabolic pathways, appears to be characterized by a wide inter-individual variability. Nevertheless, a first evaluation on stereoselectivity and bioavailability has been determined in our animal model. S-methocarbamol was generally predominant in plasma and showed no evidence of dose-dependent elimination. R-methocarbamol was also characterized by linear kinetics in the dose range of 30-220 mg/kg and showed a higher clearance with respect to the S-isomer. Both S- and R-methocarbamol showed significant first-pass effects which reduced their oral bioavailabilities to approximately 35%. The intestine appears to be responsible for most of the first-pass effect. The generality and significance of these findings remains to be confirmed in a larger population.

2.3.5. References

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2.4. Pharmacokinetics of Methocarbamol Enantiomers in Humans

2.4.1. Introduction

Methocarbamol is a centrally active muscle relaxant which has been used for decades to relieve the discomfort of painful skeletal muscle spasms. It is administered orally as a racemate. The recommended dose is 1-2 g three-four times a day [1].

Reports on methocarbamol pharmacokinetics in humans are scarce. Early studies showed that methocarbamol produced higher plasma levels and longer lasting effects in comparison with previously used muscle relaxants such as mephenesin, mephenesin carbamate and guaiafenesin [2]. The elimination half-life obtained after oral administration to healthy volunteers has been reported to be 1-2 h [3,4]. Methocarbamol pharmacokinetics do not seem to be significantly altered by haemodialysis [4].

Metabolism studies in humans showed that methocarbamol is mainly dealkylated, hydroxylated on the aromatic ring and conjugated with glucuronate and sulfate before urinary elimination. The two major metabolites, 3-(2-hydroxyphenoxy)-1,2-propanediol-1-carbamate and 3-(4-hydroxy-2-methoxyphenoxy)-1,2-propanediol-1-carbamate, are inactive. It appears that in humans less than 1 % of the dose is detected in the urine as unchanged drug [5, 6]. Human pharmacokinetic studies dealing with methocarbamol enantiomers have not been reported. The purpose of this study was

to elucidate the pharmacokinetic profiles of S- and R-methocarbamol in humans after oral administration of the racemate. Since many metabolic reactions of aromatic hydroxylation are under the control of the cytochrome P450IID6 isozyme, the population of healthy volunteers was tested for debrisoquine phenotype.

2.4.2. Experimental section

2.4.2.1. Population

Five female and five male healthy volunteers were recruited for the study. Laboratory abnormalities were not observed by routine blood and urine analyses. Data regarding age, weight and height as well as the oxidation phenotype are reported in Table 2.4.1.

2.4.2.2. Materials and methods

The single-dose study was approved by the Ethics Review Committee for Human Experimentation at the University of Alberta and written informed consent was obtained from each subject. All individuals had fasted from the night before and were asked to refrain from alcohol. They received standard meals at 4 and 8 h after administration of the drug. On the day of the study, all subjects received a single 2000 mg dose (4 tablets of Robaxin, Whitehall-Robins) of methocarbamol. Venous blood samples (5 mL) were collected in vacutainers containing heparin at 0, 0.25, 0.50, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h. Total urine output was collected for 24 h. Plasma and urine samples were analysed for methocarbamol

Table 2.4.1.- Population data for healthy volunteers evaluated for debrisoquine phenotype.

	Sex	Age	Weight,kg	Height, cm	Phenotype*
1- TP	F	26	82.3	167	EM
2- BM	F	36	65.0	180	EM
3- LD	F	30	57.5	165	EM
4- HC	F	24	64.5	165	EM
5- AS	M	35	80.4	179	EM
6- CC	M	22	79.0	191	EM
7- DF	M	29	83.4	190	EM
8- MJ	M	23	74.5	181	PM
9- JW	M	39	69.1	171	PM
10- MPM	F	28	51.0	161	PM

* EM=extensive metabolizer; PM=poor metabolizer

enantiomer levels according to a previously reported stereospecific HPLC method [7].

For determination of the phenotype, dextromethorphan was used as the probe drug [8]. Subjects of unknown phenotype were asked to take 10 ml of dextromethorphan cough syrup (Koffex, Rougier Inc.) at bedtime and provide a sample of the 8-h urinary output the following morning. All samples were stored at -20°C prior to analysis. Urine samples were tested for dextromethorphan and dextrorphan according to a reported GC method [8] slightly modified as follows. After 18 h-incubation in acetate buffer (pH 5) at 37°C with β-D-glucuronidase (Helix pomatia EC 3.2.1.31), urine samples were basified with 1N NaOH (instead of glycine buffer) to pH 11.5 and extracted with diethyl ether. A fused silica capillary column (DB-5, 7m, J&W Scientific, Rancho Cordova, CA), with temperature program, instead of a packed column in isothermal conditions, was used for the separation of dextromethorphan (retention time 3.5 min) from dextrorphan (retention time 4.6 min). Detector and injector port temperatures were set at 300°C, and oven temperature at 130 °C (rate 4°C/min to 200°C). The detection was carried out with a nitrogen-sensitive thermionic detector. The metabolic ratios were estimated by the dextromethorphan peak area / dextrorphan peak area values. A value >0.3 was used to discriminate between poor metabolizers and extensive metabolizers. Our poor metabolizers showed values of 1.0, 8.1, and 12.3, whereas for the extensive metabolizers the ratio was always <0.1

2.4.2.3. Data analysis

Area under the plasma concentration-time curve (AUC) was calculated by the trapezoidal rule and extrapolated to infinity where appropriate. Half-life ($t_{1/2}$) was estimated using linear regression of the terminal log-linear phase. Oral clearance (CL_o) was evaluated by the formula Dose/AUC. Urinary excretion of the unchanged enantiomers was expressed as percentage of the dose (X_u^∞). Significance of the observed differences was evaluated by the paired Student's t-test. Level of significance was set at $p<0.05$.

2.4.3. Results

Pharmacokinetic parameters are reported in Table 2.4.2. In EMs S-methocarbamol AUCs and C_{max}s, were significantly higher than those of R-methocarbamol. As a consequence, oral clearance of S-methocarbamol was significantly lower than that of R-methocarbamol. Significant differences were not observed between enantiomers in half-life and t_{max}. Statistical analysis was not performed between extensive and poor metabolizers data because of the limited size of the populations. However, it was observed that in the poor metabolizer subjects #9 and #10 both enantiomers showed remarkably higher (about twice as high) AUCs compared to the values obtained for the extensive metabolizers. Subject # 8, however, who was also classified as a poor metabolizer, showed plasma levels of S- and R-methocarbamol that were comparable to those of the extensive metabolizers. Urinary recovery of the enantiomers, as

Table 2.4.2.- Pharmacokinetic parameters in 10 healthy volunteers after oral administration of a single 2 g dose of racemic methocarbamol.

Subject #	$AUC_{(0-\infty)}$, mg L^{-1} h			$t_{1/2}$, h		Cmax, mg/L			tmax, h	
	S	R	Ratio,S/R	S	R	S	R	Ratio, S/R	S	R
EMs										
1	36.4	29.4	1.2	1.4	0.6	18.3	17.8	1.0	1.0	1.0
2	38.6	17.0	2.3	1.1	0.2	11.7	6.6	1.8	1.5	1.5
3	98.1	34.7	2.8	0.9	1.3	13.2	10.3	1.3	1.5	1.5
4	61.0	29.5	2.1	0.9	2.5	13.9	10.1	1.4	3.0	1.5
5	27.6	18.0	1.5	0.5	0.3	13.3	13.4	1.0	1.5	1.5
6	27.0	18.2	1.5	1.4	1.8	13.2	11.7	1.1	1.0	1.0
7	13.1	12.7	1.0	1.0	0.7	3.3	3.6	0.9	3.0	3.0
Mean±SD	43.1±28.3	22.8±8.3*	1.8±0.6	1.0±0.3	1.1±0.8	12.4±4.5	10.5±4.6*	1.2±0.3	1.8±0.8	1.6±0.7
PMs										
8	25.8	23.2	1.2	0.6	0.5	11.9	9.7	1.2	1.0	1.0
9	128.3	75.6	1.7	3.7	1.7	34.9	29.0	1.2	1.0	1.0
10	123.6	124.6	0.8	1.7	0.4	28.5	25.7	1.1	2.0	2.0
Mean±SD	92.6±57.9	74.5±50.1	1.3±0.4	2.0±1.3	0.9±0.7	25.1±11.9	21.5±10.3	1.2±0.06	1.3±0.6	1.3±0.6

* $p<0.05$ (paired t-test) significantly lower than the corresponding value for S-methocarbamol.

Table 2.4.2.- cont.

Subject #	CL_o, L/h		X_u^∞, % dose		
	S	R	S	R	S/R
EMs					
1	27.5	34.0	0.4	0.3	1.3
2	25.9	58.8	0.3	0.3	1.0
3	10.2	28.8	1.4	1.0	1.4
4	16.4	33.9	0.9	0.7	1.3
5	36.2	55.6	2.1	1.9	1.1
6	37.0	54.9	0.3	1.3	0.9
7	76.3	78.7	0.9	1.0	0.9
Mean±SD	32.8±21.5*	49.2±17.9	0.9±0.7	0.9±0.5	1.1±0.2
PMs					
8	38.7	43.1	5.7	6.1	0.9
9	7.8	13.2	0.2	0.1	2.0
10	8.1	8.0	1.9	1.5	1.3
Mean±SD	18.2±17.7	21.4±18.9	2.6±2.8	2.6±3.1	1.4±0.6

* $p<0.05$ significantly lower than the corresponding value for R-methocarbamol.

unchanged S- and R-methocarbamol, in extensive metabolisers did not exceed 2.1 and 1.9 % of the administered dose respectively . In poor metabolisers up to 5.7 % of the dose was eliminated as S-methocarbamol and up to 6.4 % as R-methocarbamol. Results are summarized in Table 2.4.2. Plasma concentration-time profiles of one extensive and one poor metabolizer are depicted in Fig. 2.4.1. The rest of the curves are reported in Appendix I.

2.4.4. Discussion

Variability in drug metabolism and disposition is dependent upon different individual characteristics such as age, diseases, environmental pollutants and genetic polymorphism; their influence on the pharmacokinetic profile of a drug is sometimes difficult to determine especially if the drug presents a complicated pattern of metabolism. The genetic polymorphism of debrisoquine metabolism is one of the best studied examples of genetic variability in drug response. In the Caucasian population, 5 to 10% of individuals are classified as "poor metabolizers". The human P-450 enzyme catalysing debrisoquine oxidation, i.e. P450IID6, is absent in poor metabolizers. When a drug (or an active metabolite) is a substrate for this enzyme and the extent of the elimination via alternative pathways such as non oxidative metabolism, excretion of the unchanged drug and metabolism by different P-450 isozymes, is not significant, the lack of the P450IID6 isozyme becomes evident in the pharmacokinetic profile. As a consequence, pharmacokinetic parameters in EMs are significantly different from those of the PMs.

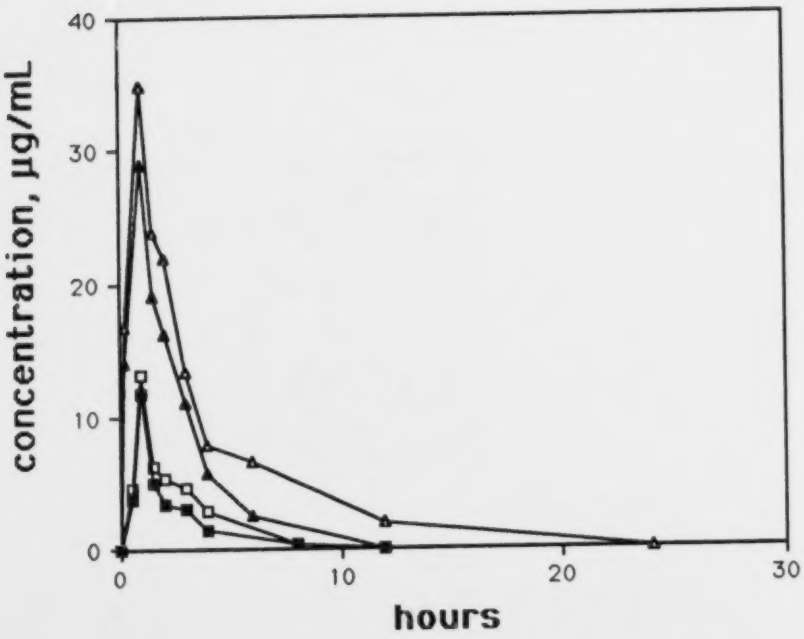

Figure 2.4.1 Plasma concentration time profiles of S- and R-methocarbamol in one PM [(△) S-methocarbamol, (▲) R-methocarbamol] and in one EM [(□) S-, (■) R-methocarbamol].

Non-stereoselective studies on the racemate have shown that methocarbamol is metabolized in humans [6] by O-demethylation and aromatic hydroxylation. These pathways are under the control of the P450IID6 isozyme for basic drugs, but specific studies have notbeen conducted to investigate co-segregation of methocarbamol with the debrisoquine phenotype.

The purpose of this study was to elucidate the pharmacokinetic pattern of methocarbamol enantiomers in humans after oral administration of the racemate. The debrisoquine phenotypes of healthy volunteers were determined in order to investigate the effect of the impairment of oxidative metabolism on the pharmacokinetic profiles of S- and R-methocarbamol.

Results showed significant stereoselectivity, with S-methocarbamol always predominant in the plasma of the EMs. S-methocarbamol AUCs were significantly higher than the corresponding AUCs for R-methocarbamol. Similarly C_{max} was significantly higher for S-methocarbamol compared to C_{max} for R-methocarbamol. Half-lives, however, seemed to be comparable. These observations may indicate stereoselective first-pass metabolism. Differences in t_{max} were not observed and therefore it is likely that the rate of absorption would be similar for the two enantiomers. Stereoselectivity was not observed in urinary excretion of the unchanged enantiomers.

Subjects #9 and #10 (PMs) showed considerably higher AUCs for both enantiomers in comparison with the corresponding values in EMs. However, subject #8 who was also classified as a PM, showed plasma levels that were comparable to those of the EMs; interestingly

this subject showed the highest urinary recovery of both unchanged enantiomers (5.7 and 6.1 % for S- and R-methocarbamol, respectively). The urinary excretion of unchanged enantiomers otherwise appeared to be a minor pathway of elimination, being generally less than 1% of the dose. Only one of the PMs showed a prolonged half-life for S-methocarbamol (subject #9), while the other two had values comparable to those of the EMs. Steroselectivity in PMs was similar to that of EMs with S-methocarbamol usually predominant in plasma (higher C_{max}). Since t_{max}s of both enantiomers, were not significantly different between EMs and PMs the rate of absorption is also expected to be similar between EMs and PMs. The similarity of the patterns between EMs and PMs suggests that the more elevated plasma levels observed in two of the PMs may be due to a reduced first-pass effect. The fact that one of the poor metabolizers showed pharmacokinetic patterns that were superimposable to those of the EMs can be explained by the possibility that in some individuals the P450IID6 oxidation is not a major pathway for methocarbamol enantiomer metabolism.

In summary, in the present study the pharmacokinetic parameters of S- and R-methocarbamol have been determined in humans after the administration of a 2 g dose of the racemate. Significant stereoselectivity was observed, with S-methocarbamol generally predominant in plasma. The effect may be attributed to stereoselective first-pass metabolism. On the basis of our observations a final conclusion on the co-segregation of methocarbamol with the debrisoquine phenotype cannot be drawn. However, the elevated plasma levels observed in two PMs indicates

that S- and R-methocarbamol could be substrates for the P450IID6 isozyme despite the fact that methocarbamol is not a basic drug. The generality of these observations remains to be verified in a larger population. Furthermore the clinical significance of the stereoselectivity involved in methocarbamol pharmacokinetics is highly dependent on whether the two enantiomers exihibit different pharmacological properties.

2.4.5. References

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2.5. Conclusion

The stereospecific analytical method now reported is the first one described for the separation of methocarbamol enantiomers. The application of this assay to the study of the pharmacokinetics of S- and R-methocarbamol in animal models and humans produced the first data on this subject.

Stereoselectivity was revealed by examining the plasma of rats after intravenous administration of the racemate; in plasma S-methocarbamol was predominant. Dose-dependency was not detected in the range of dosage used (30-220 mg/kg) for both enantiomers. S- and R-methocarbamol showed significant first pass metabolism which reduced their oral availability to about 35%. The most probable site of first pass metabolism appeared to be the intestine. These findings are not in complete agreement with a previous non-stereoselective report on the pharmacokinetics and bioavailability of methocarbamol in the rat [1] which showed dose-dependent kinetics in the range 15-150 mg/kg and a bioavailability of 70-83 %; other reported pharmacokinetic parameters were comparable. The reason for these discrepancies could be found in the different experimental approach used in the two studies. In the mentioned publication [1] the plasma concentration-time curves were determined by averaging plasma concentrations obtained from each of four rats sacrificed at different intervals after administration of the drug. In the current study plasma profiles were followed in each animal. Surgical incannulation of the jugular vein of the animals was performed and blood samples were withdrawn at different

intervals. As a consequence, current data, which were reported as means of the pharmacokinetic parameters calculated separately for each rat, showed a greater inter-animal variability but were, nevertheless, more representative of the real biological situation. The variability decreased the power of the statistical analysis and this was probably the reason why only a trend toward lower clearances and longer half-lives was observed at the highest level of dosage, without reaching statistical significance. Lower estimation of the biovailability when compared with the values previously reported can be explained by a reduction in absorption as a consequence of the surgical cannulation [2]. However, a reduced biovailability could actually partially explain the high doses that are required to obtain therapeutic effectiveness from this low-potency muscle relaxant. A report [3] describing the necessity for administering oral doses as high as three times the intraperitoneal dose to obtain the same pharmacological response in animal models, as well as evidence of low bioavailability in the horse [4], support this hypothesis.

In humans, S-methocarbamol was also predominant in plasma after oral administration of a 2 g dose of the racemate, confirming that the rat can be considered a good model. Higher AUC values for S-methocarbamol with respect to the R-isomer could be explained by slight differences in first pass effects between enantiomers because significant changes in half-life were not evident. Two poor metabolizers of debrisoquine were observed to have elevated plasma levels of methocarbamol enantiomers relative to the levels observed in extensive metabolizers, suggesting that methocarbamol could be a substrate for the P450IID6 isozyme.

In order to complete the investigation on methocarbamol enantiomers, more extensive studies on the pharmacodynamics should be conducted. A preliminary investigation showed that there were no significant differences in muscle relaxant activities when the enantiomers were evaluated in mice using a "traction" test [5]. Moreover a controlled evaluation of bioavailability as well as a detailed study on intravenous pharmacokinetics in humans would also be of interest. Further investigations could also include studies of *in vitro* and *ex vivo* metabolism.

2.5.1. References

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3. General conclusions and future perspectives

The possibility of expanding investigations on new and old drugs is strictly related to the development of analytical techniques with sufficient sensitivity, versatility and applicability to allow a complete study of the biological fate of pharmaceuticals in animal models and humans. Stereoselectivity is an important aspect of drug utilization that was neglected for decades. It is now clear that biological systems recognise optical isomers as different compounds and can interact differently with them. It is surprising that only recently has such an observation been introduced into pharmaceutical research when as long ago as 1860 Pasteur proposed that asymmetry was "perhaps the only well marked line of demarcation that can at present be drawn between the chemistry of dead matter and the chemistry of living matter" [1].

Numerous new optically pure reagents and mobile phase additives for indirect chromatographic resolution, and chiral columns for direct separation have become available on the market in recent years. These have made easier the task of developing stereospecific analytical methods but there is no guarantee of success and chromatographic separation of enantiomers can often be considered more an "art" than a technique. Flecainide and methocarbamol are two examples of "difficult" drugs. With flecainide, steric hindrance makes the derivatization of the secondary amine moiety difficult. Problems related to asymmetric induction were also observed in the absence of a heating step during derivatization. The natural

fluorescence of the molecule was also quenched by derivatization, with consequent loss of detection sensitivity.

Methocarbamol is a relatively unreactive molecule with only the alcohol group amenable to derivatization. Reaction with NEIC at this site does not occur at room temperature; a long incubation time (12 hours at 85 °C) is necessary to obtain a derivatization yield sufficient for a sensitive assay. The natural fluorescence of the reagent (NEIC) seemed to be quenched by the reaction and a fluorescence detector did not improve the sensitivity compared to UV. A valid alternative may have been the use of a chiral column. "Pirkle" columns with a dinitrobenzoyl-derivatized phenylglycine packing might have the appropriate structure for the separation of S- and R-methocarbamol, because of their stuctural similarity to β–blockers [2]. Methocarbamol has been resolved, without derivatization, on an S-t-leucine/R-1-(α-naphthyl) ethylamine covalent urea column using a mobile phase consisting of hexane:1,2-dichloromethane: ethanol-trifluoroacetic acid (60:35:5; ethanol-TFA proportion 20:1) (R_t of enantiomers, 10.63 and 11.31 min; α=1.09) [3].

The application of stereospecific methods developed for racemic flecainide and methocarbamol provides a means of expanding the knowledge base on these two drugs. The pharmacokinetic profiles of their enantiomers have been delineated and several observations have been made with respect to their biological fate. The relevance of these findings, in terms of clinical utilization of the drugs, might not be dramatic: stereoselectivity does not seem to be very pronounced in either drug. The parallel pharmacokinetic patterns of flecainide enantiomers together with their equiactivity and

equipotency, comfirm that this drug is not a good candidate for single enantiomer administration, unless differences in long term toxicity can be detected between enantiomers. Methocarbamol is generally a non-toxic drug and the pharmacokinetic properties of its enantiomers do not differ dramatically, even though the S-enantiomer is generally predominant in plasma after administration of the racemate. A preliminary report on the muscle relaxant properties of the enantiomers in mice did not reveal any substantive differences in potencies between S- and R-methocarbamol [4]. As a consequence, unless it is shown that the relative efficacies of the enantiomers drastically differ in humans, methocarbamol can probably be administered safely as the racemate.

Nevertheless, the general importance of stereoselectivity in drug action and disposition should never be underestimated and knowledge should always be pursued even before a practical application can be foreseen.

3.1. References

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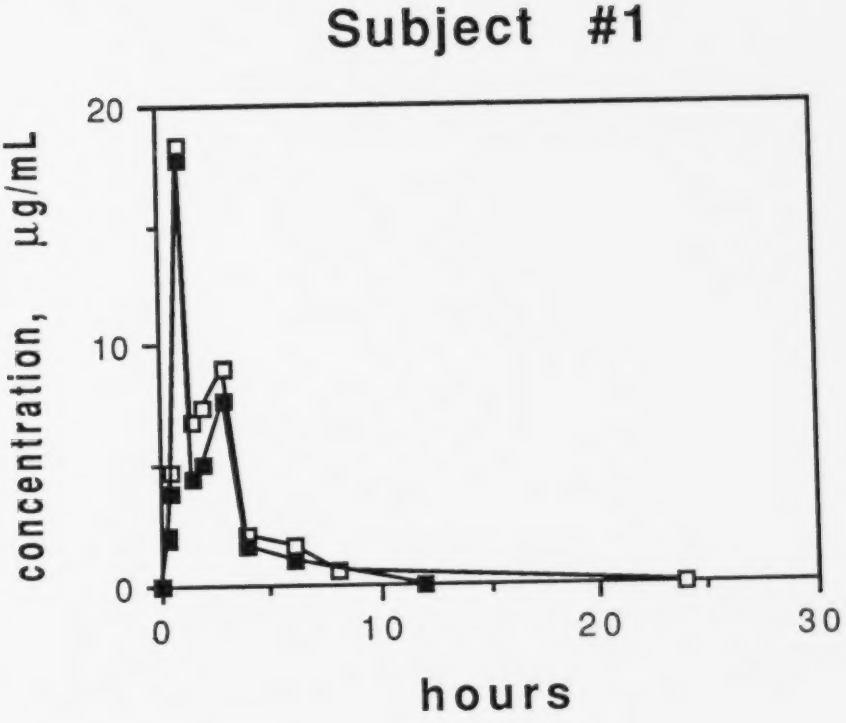
APPENDIX I

Plasma concentration - time profiles of S- (□) and R-methocarbamol (□) in 10 healthy subjects after oral administration of a 2 g dose of racemic methocarbamol.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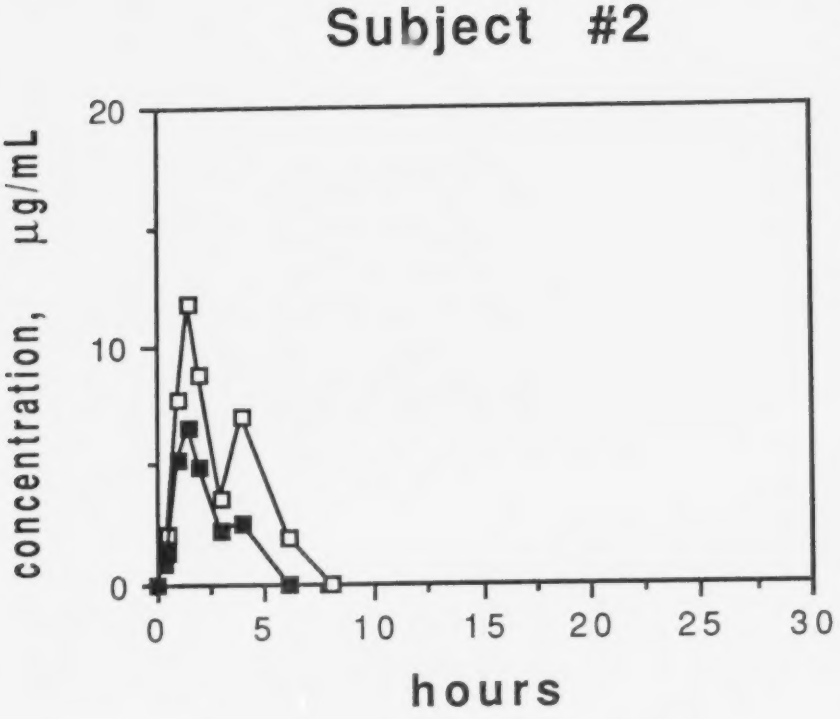
0
10
20
30
hours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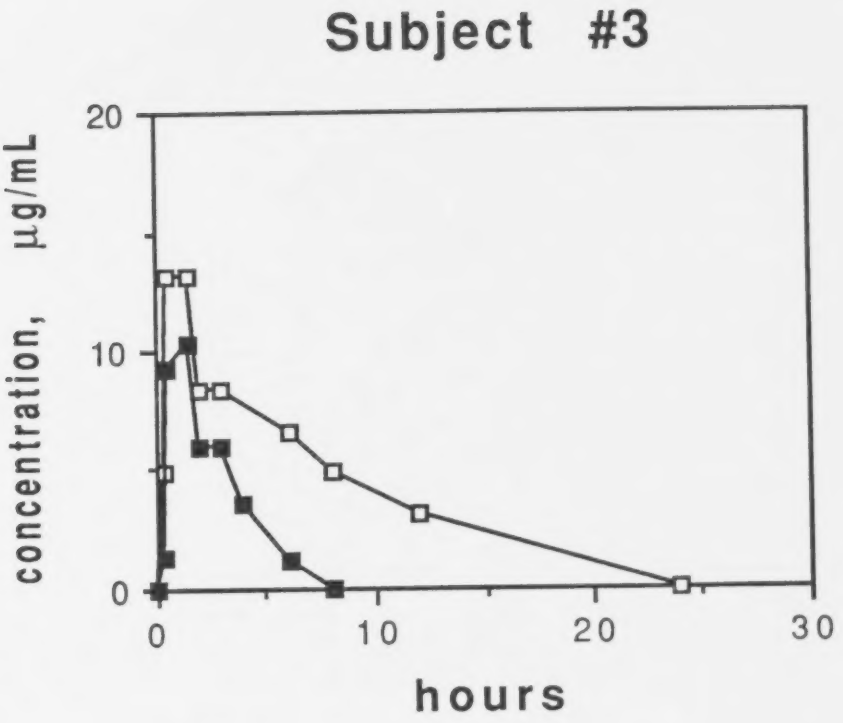
10
15
20
25
30
hours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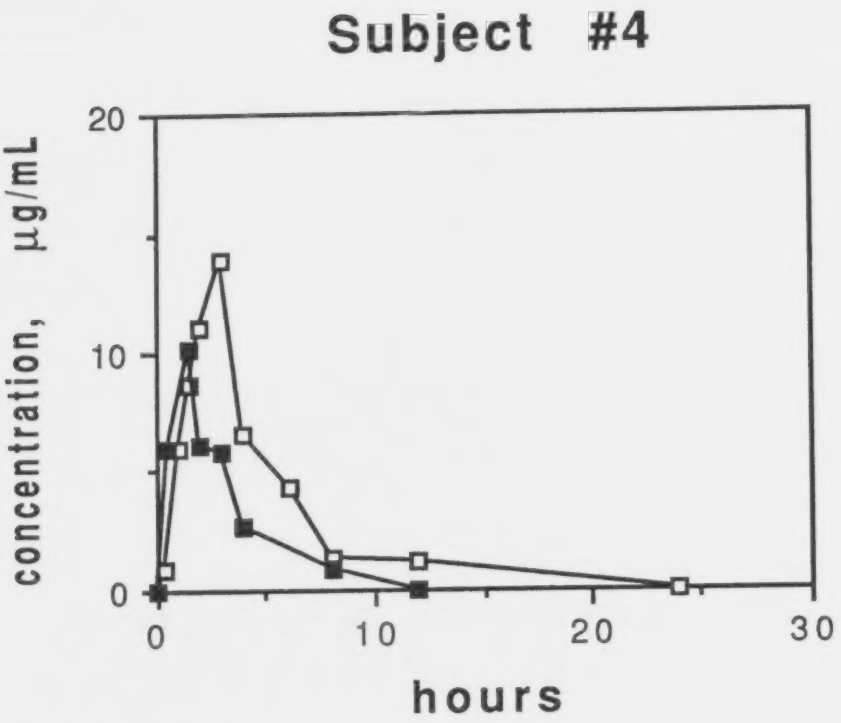
0
10
20
30
hours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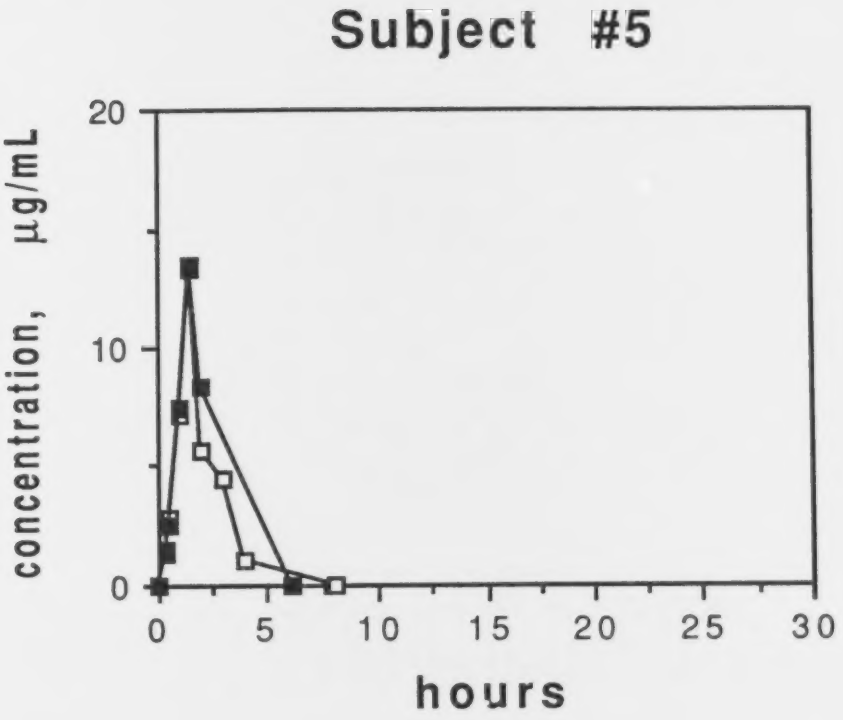
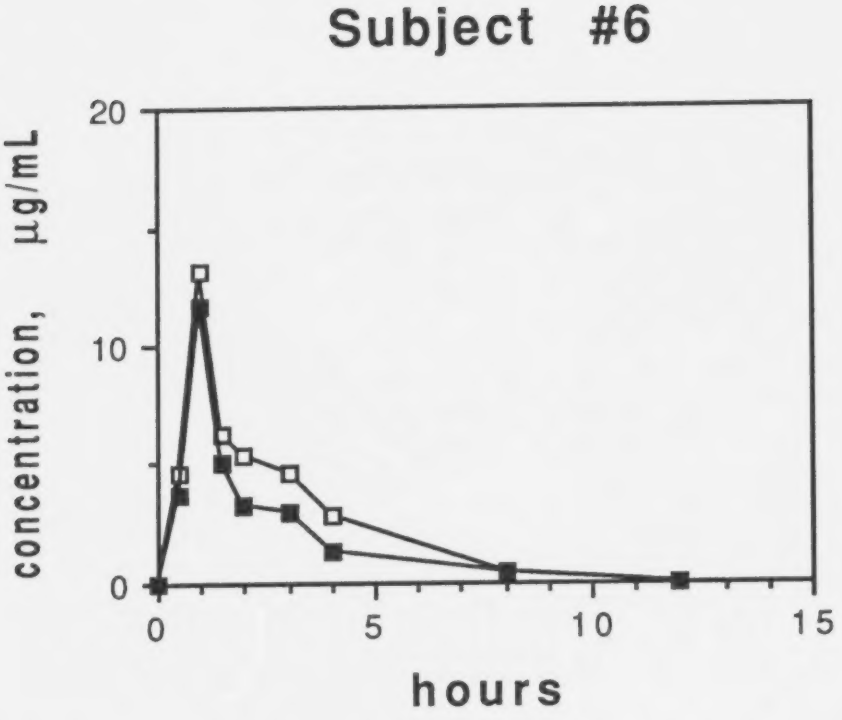
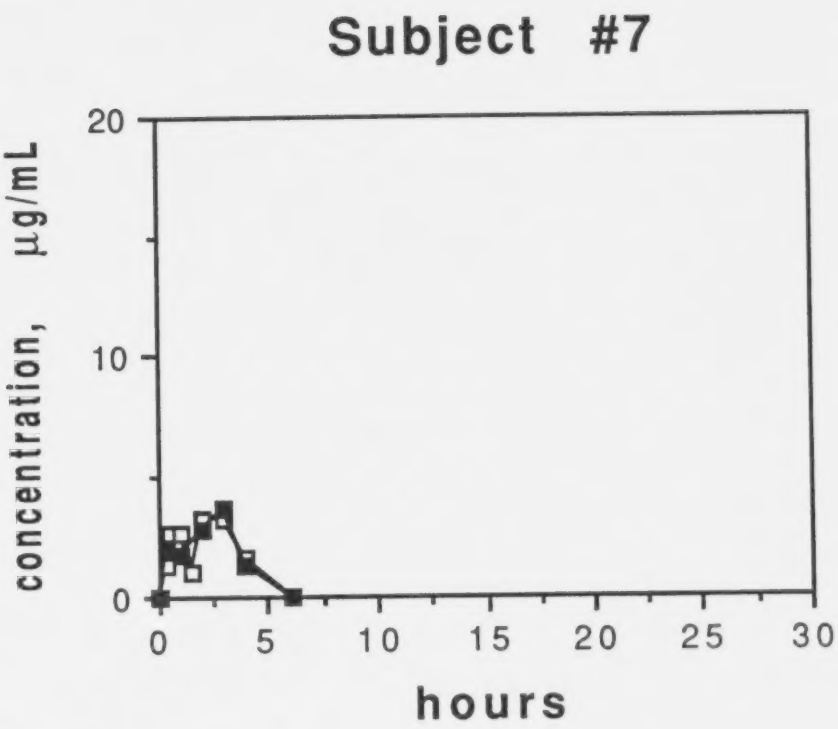
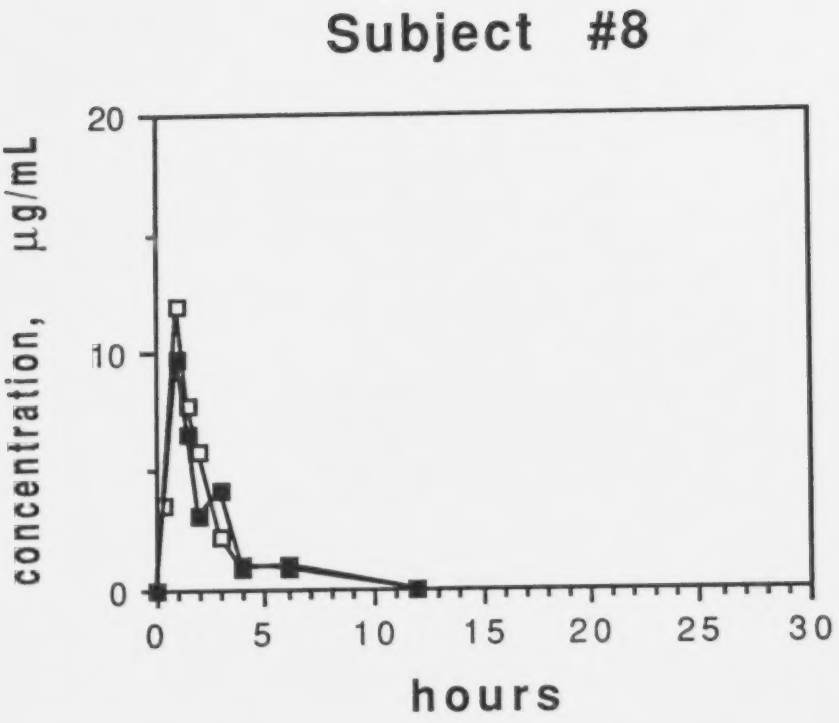
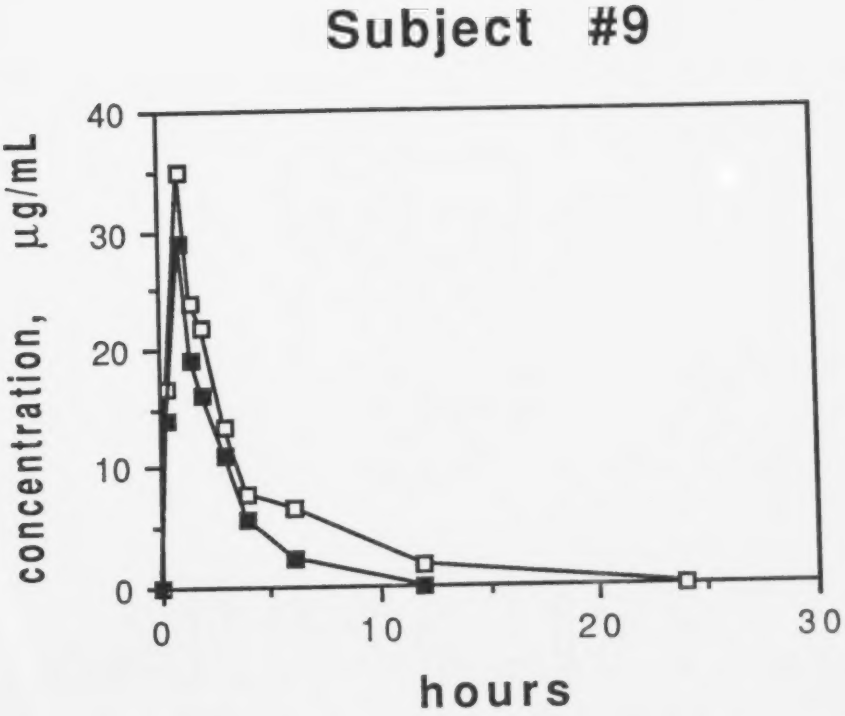
0
10
20
30
hours

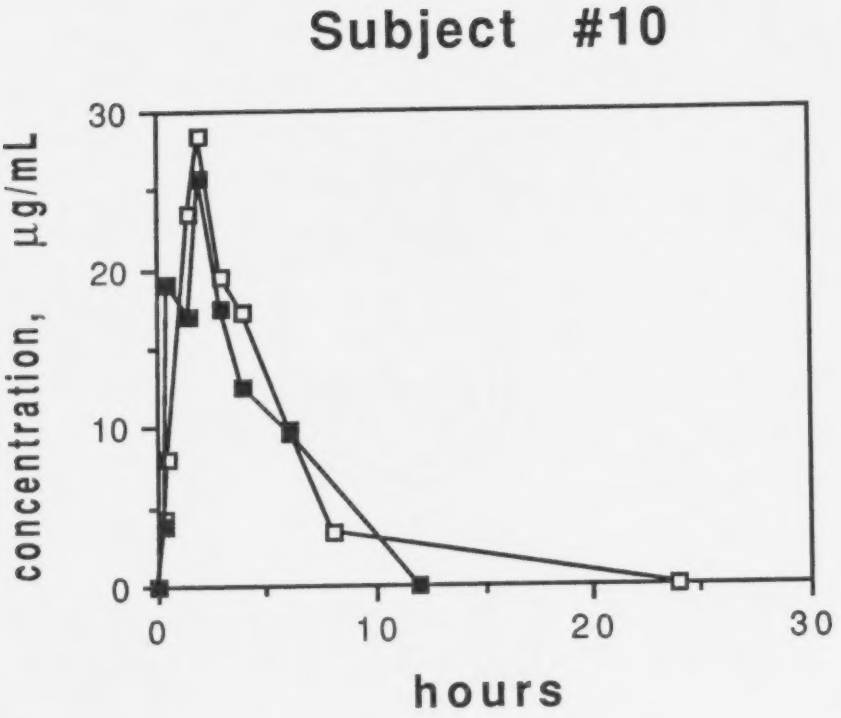

Subject #5
concentration, µg/mL
20
10
0
0
5
10
15
20
25
30
hours


Subject #6
concentration, µg/mL
20
10
0
0
5
10
15
hours


Subject #7
concentration, µg/mL
20
10
0
0
5
10
15
20
25
30
hours


Subject #8
concentration, µg/mL
20
10
0
0
5
10
15
20
25
30
hours


Subject #9
concentration, µg/mL
40
30
20
10
0
0
10
20
30
hours


Subject #10
concentration, µg/mL
30
20
10
0
0
10
20
30
hours